DNA Phenotyping:

The Prediction of Human Pigmentation Traits from Genetic Data

Susan Walsh

The work presented in this thesis was conducted at the Department of Forensic Molecular Biology, Erasmus MC University Medical Center Rotterdam in collarboration with various academic and forensic partners, most notably the Section of Forensic Genetics, Institute of Forensic Research/Department of Genetics and Evolution, Jagiellonian University, Kraków, Poland. This work was funded by the Netherlands Forensic Institute (NFI), the Erasmus MC University Medical Center Rotteram, and received additional support by a grant from the Netherlands Genomics Initiative (NGI)/Netherlands Organization for Scientific Research (NWO) within the framework of the Forensic Genomics Consortium Netherlands (FGCN). The group in Kraków was supported by a grant from the Polish Ministry of Science and Higher Education (No. ON301115136 fund of science, years 2009–2012). The EUREYE study, which provided materials to this work was supported by the European Commission 5th Framework (QLK6-CT-1999-02094), the Macular Disease Society UK and the Estonian Ministry of Education and Science (target funding SF0940026s07).

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DNA Phenotyping:
The Prediction of Human Pigmentation Traits from Genetic Data

DNA Fenotypering:
De Voorspelling van de Mens Pigmentatie Kenmerken van genetische gegevens

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ter verkrijging van de graad van doctor aan de
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Prof.dr. H.G. Schmidt

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	Prof.dr. L. Roewer

Stellingen

Behorende bij het proefschrift

DNA Phenotyping: The Prediction of Human Pigmentation Traits from Genetic Data

1. DNA phenotyping has the ability to unlock cold cases in forensics, missing-persons cases and reveal our ancestors physical appearance.
2. The IrisPlex system can predict human blue and brown eye colour with > 94% accuracy. (This thesis)
3. The HIrisPlex system can predict human hair colour categories blond, brown, black and red with an average accuracy of 79%. (This thesis)
4. Validation of the IrisPlex and HIrisPlex systems ensure their suitability for forensic applications. (This thesis)
5. Quantitative pigmentation phenotyping has the potential to find new underlying genes. (This thesis)
6. Moving eye and hair colour DNA prediction from current categorical levels to future continuous levels will provide most detailed outcomes. (This thesis)
7. The intronic HERC2 rs12913832 SNP region functions as an enhancer regulating OCA2 transcription. (Visser 2012)
8. The association effect of a SNP with a phenotype does not automatically equal a predictive effect.
9. Variation in human eye and hair colour is of European origin and was shaped by sexual selection i.e. mate choice preferences [Frost 2006]
10. Human eye and hair colour variation within Europe is older than 5300 years. (This thesis)
11. There's no gene for fate. [GATTACA, 1997]

PUBLICATIONS AND MANUSCRIPTS BASED ON THE STUDIES DESCRIBED IN THIS THESIS

Chapter 2.1

S. Walsh, F. Liu, K. N. Ballantyne, M. van Oven, O. Lao, M. Kayser, IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Science International Genetics 5 (2011) 170-180.

Chapter 2.2

S. Walsh, A. Lindenbergh, S. B. Zuniga, T. Sijen, P. de Knijff, M. Kayser, K. N. Ballantyne, Developmental validation of the IrisPlex system: Determination of blue and brown iris colour for forensic intelligence. Forensic Science International Genetics 5 (2011) 464-471.

Chapter 2.3

S. Walsh, A. Wollstein, F. Liu, U. Chakravarthy, M. Rahu, J. H. Seland, G. Soubrane, L. Tomazzoli, F. Topouzis, J.R. Vingerling, J. Vioque, A.E. Fletcher, K.N. Ballantyne, M. Kayser, DNA-based eye colour prediction across Europe with the IrisPlex system. Forensic Science International Genetics 6 (2012) 330-340.

Chapter 2.4

A. Wollstein, **S. Walsh**, F. Liu, S. Bohringer, M. Kayser, A new approach for quantifying human pigmentation from digital imagery: improved eye colour phenotyping enhances genetic association and prediction, Draft prepared.

Chapter 3.1

S. Walsh, F. Liu, A. Wollstein, L. Kovatsi, A. Ralf, A. Kosiniak-Kamysz, W. Branicki, M. Kayser, The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. Forensic. Science International: Genetics 7 (2013) 98-115.

Chapter 3.2

S. Walsh, L. Chaitanya, L. Clarisse, L. Wirken, J. Draus-Barini, L. Kovatsi, T. Sijen, P. de Knijff, W. Branicki and M. Kayser, Developmental Validation of the HIrisPlex System: DNA-based eye and hair colour prediction for forensic usage. Forensic Science International: Genetics, Submitted.

Chapter 4.1

S. Walsh, A. Wollstein, S. Wilde, L. Kovatsi, A. Kosiniak-Kamysz, E. Kaiser, W. Branicki, J. Burger and M. Kayser, Human eye and hair colour variation is older than 5,300 years. Pigment Cell & Melanoma Research, Submitted.

Chapter 4.2

J. Draus-Barini, **S. Walsh**, E. Pośpiech, T. Kupiec1, H. Głąb, W. Branicki & M. Kayser. Bona fide colour: DNA prediction of human eye and hair colour from ancient and contemporary skeletal remains. Investigative Genetics 2013 4(3).

 magination is the beginning of creation.

You imagine what you desire;

You will what you imagine;

And at last you create what you will.

George Bernard Shaw

To Mike,

Mam & Dad,

and my brothers,

especially Mark.

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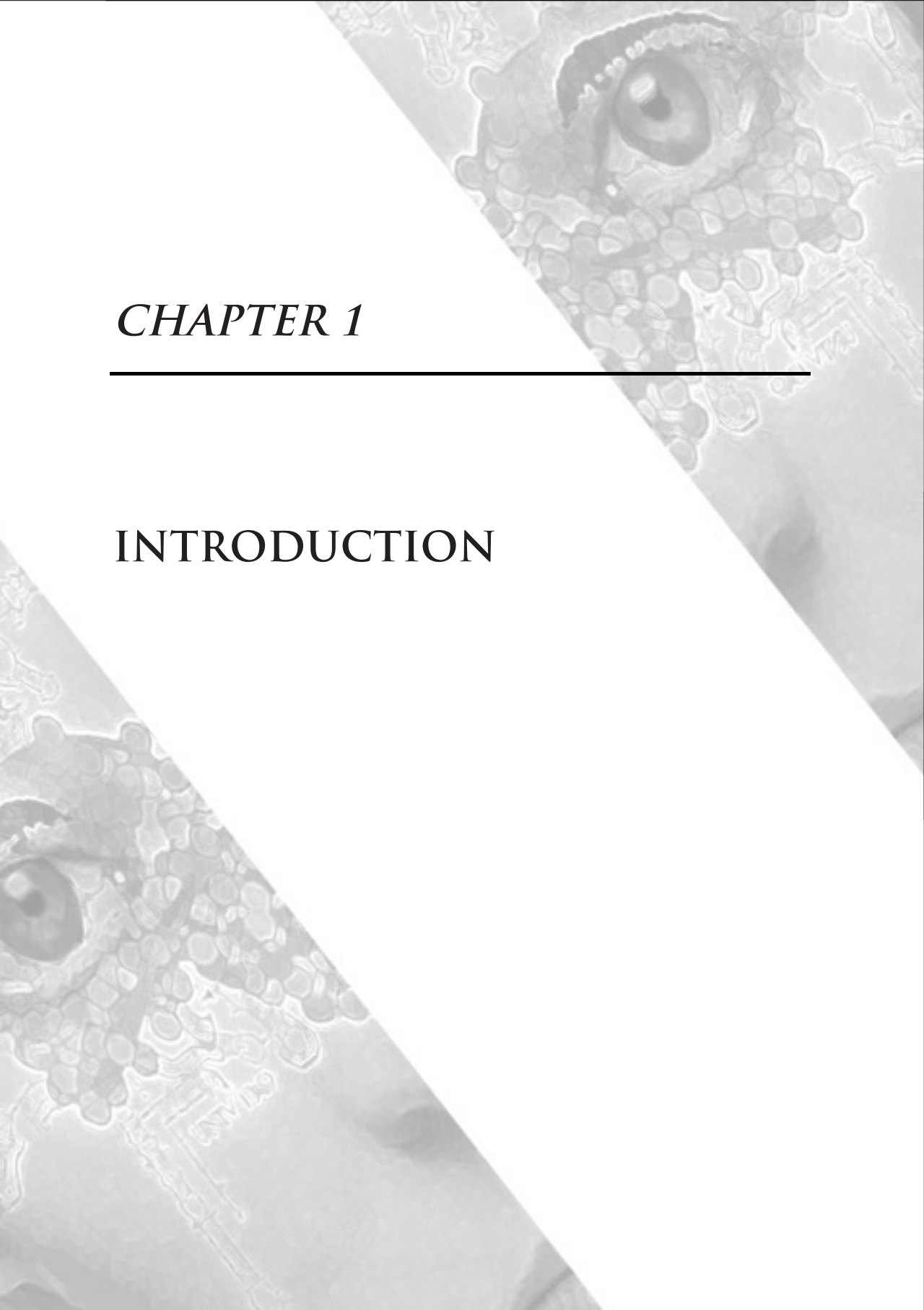


CHAPTER 1

INTRODUCTION

The definition of a phenotype is the observable physical or biochemical characteristics of an organism, as determined by both genetic makeup and environmental influences. Phenotyping is the ability to assign these characteristics to an organism based on certain measurable parameters. In the case of DNA phenotyping, it is limited to the sole use of genetic information such as DNA to determine a phenotype. Advances in medical and human genetics have provided a more detailed understanding of the impact of genetics on phenotypes, often due to large collaborative research efforts. Major prerequisites for doing these were international efforts to map and catalogue human genetic information. This includes the human genome project and their publication of a reference human genome sequence [1], the international HAPMAP project (http://hapmap.ncbi.nlm.nih.gov/), along with a reference database from the ENCODE project of 3.2 million human genome SNPs [2] to name a few. There are now more than 38 million validated SNPs in the NCBI database (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) that is publically accessible. This has led to the development of SNP microarrays covering hundreds of thousands, and currently up to 1 million SNPs of genome-wide coverage. Their use lies in parallel genotyping and genome-wide association studies (GWAS) performed on DNA samples from individuals, in considerable numbers, for which phenotypes have also been measured. There has been a lot of knowledge obtained from candidate gene studies and more recently from exome or whole genome sequencing. However, studies using next generation sequencing technologies (NGS) have delivered and will continue to deliver even more information on the genes most likely involved in a particular phenotype of interest. Highly associated phenotypic SNPs that are obtained from these studies are then

tested for their effect in predicting a phenotype, solely based on the genetic information available from these DNA markers. The area of DNA phenotyping has an application in several different fields, such as personalized medicine and forensic science.

Personalised medicine (PM) and the use of predicting cancer and other human diseases from DNA has been developing over the last few years [3, 4] with great promise. It would be of great benefit to know the genetic risk involved in already having the disease, or even prevent the chances of developing a disease by knowing the environmental factors that trigger it in the future. This DNA predictive element of personalized medicine has drawn immense critical attention among the scientific community [5]. However, consumer genetic companies offering genome-wide tests to assess disease risk prediction solely based on DNA, reap from the public's more favourable and uneducated attitude. Cancer genetics in particular has led to the development of some of the first PM prediction tests for hereditary breast-ovarian cancer by testing for disease-causing mutations in the *BRCA1* and *BRCA2* genes [6]. In certain circumstances, a mutation that results in a disease is often recessive, so the individual would need both mutant alleles for the disease to be expressed phenotypically. If this is the result of more than one locus with or without environmental triggers it is the result of multifactorial inheritance, and is therefore a complex trait. As with many complex traits, the amount of heritability cannot all be explained by genetics and there are bigger challenges such as understanding the environmental influences to factor in. An additional goal of personalized medicine, once a disease risk or background susceptibility to a disease is identified e.g. from DNA, lies in tailored-drug therapy, a field also referred to as pharmacogenetics [7].

Another aspect of DNA phenotyping that is similar in design to disease risk prediction, although quite different in its final use, is the prediction of externally visible characteristics (EVCs) solely from DNA. The principle is the same, however instead of a disease-causing phenotype that is found through phenotype distinctions of case versus control, a physical appearance trait is defined as the phenotype of interest, i.e. eye colour. Most if not all human EVCs are multifactorial complex traits and it is the interaction of several genes among themselves and the environment that define the phenotype [8]. Similar to common diseases, the phenotype complexity (both genetically and environmentally) troubles the search for an underlying genetic basis, which can often only be solved by analysing large numbers of samples. The explanation for this is due to the individual phenotypic effect for each DNA marker being so small.

Human EVC prediction in DNA phenotyping has applications in evolutionary biology, anthropology and forensics. For evolutionary biology it is relevant to understand how and why humans have adapted during evolution and one of the most obvious variable phenotypes are the pigmentation traits, eye, hair and skin colour. Some of the positive selection forces at work that shape these pigmentation traits have been proposed as sexual selection of human mate choice [9] for hair and eye colour, or adaptation to UV radiation (UVR) exposure in terms of skin colour [10]. It is these research areas that help shed light on how our ancestors evolved and why [11]. There are several reasons why phenotyping and an explanation of the genetics and evolutionary history of pigmentation is important. One of the main informative values is that data can be used to help improve model parameters used in population

evolutionary genetics. These studies help trace back modern human genetic data to explain the forces that impacted on our ancestors that led to changes in the ancestral phenotypes. For instance, going from black hair, brown eyes and dark skin, to the frequencies of lighter pigmentation phenotypes seen in Europe today. Usually, such knowledge is derived from modern populations using computer simulations but because these approaches face statistical limitations, it is advantageous to use direct genetic evidence via ancient DNA (aDNA) analysis of fossil remains from different time scales. In this instance, the field of aDNA work is currently emerging due to promising DNA technologies such as NGS [12].

In biological anthropology, its relevance usually lies in the molecular and genetic basis of non-disease human phenotypes where EVC prediction provides a particular fascination. Unfortunately, some EVCs such as skin colour have been misused in the past by anthropologists, and especially by non-experts to underline racism claims. However, with ancestry knowledge now derived from DNA that argues against race theory, the field of appearance biology has regained interest [13]. Furthermore, appearance phenotypes such as body height [14] can also be useful as a model in the understanding of the genetic architecture of complex traits in general. This may also help in the comprehending of complex disease traits. Understanding the strong signatures of adaptation between populations in the variation of human pigmentation, especially skin colour, also has relevance in health care. It may help determine the risk factors for certain types of skin cancer that may be associated with these evolutionary adaptive genes [15].

DNA phenotyping as an application in forensic genetics encompasses the ability of a forensic investigator to predict externally

visible traits of an individual, using molecular analyses from biological samples. These sources can include samples left at a crime-scene to missing person's identification. In current forensic practice, short tandem repeat (STR) DNA typing is the preferred method of identification of a donor from biological material found at a crime scene. However, if this unknown STR profile does not match that of a known suspects profile or any from a criminal DNA database, and if no other evidence is present, the investigation has lost any leads to pursue. In cases such as this, the investigation can now turn towards alternative DNA analysis methods, a term called 'DNA intelligence'. It allows the prediction of externally visible characteristics (EVCs) using informative DNA markers, which can be described as forensic DNA phenotyping (FDP), the use of which has started to become a new and exciting area of investigation [16]. This DNA intelligence information from DNA phenotyping may be used as a 'biological witness' in multiple circumstances from routine casework to disaster victim and missing person identification if conventional identification procedures fail. For crime scenes, FDP is expected to reduce the pool of potential suspects and therefore concentrate police investigations and guide them to find the 'unknown' perpetrators [16]. By using developmentally validated tools that pass the strict guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM), accredited forensic laboratories can use these new DNA profiling methods combined with knowledge of pigmentation traits such as eye and hair colour to provide the first glimpse into the physical appearance perception of an individual which will enable the threading of a police investigation net for more leads. Research in the area of pigmentation genetics such as eye and hair colour has strived in the last few years with

potential applications in forensic DNA phenotyping as they are one of the first reported descriptions in eye-witness statements.

The biology of human skin, eye, and hair colour

Melanin is the main pigment of eye, hair and skin colour. It is a mixture of biopolymers synthesised in melanocytes that are located in the basal layer of the epidermis, the hair bulb, and the iris [15]. It is not the number, distribution or density of the melanocyte cells themselves that account for phenotypic differences in pigmentary traits, but the regulation of the process of melanogenesis [17]. Tyrosinase (*TYR*) is the key enzyme in melanogenesis and mediates the first steps in melanin synthesis [18], which is the oxidation of its substrate tyrosine to form an intermediate compound called DOPAquinone [19]. From this, two major types of

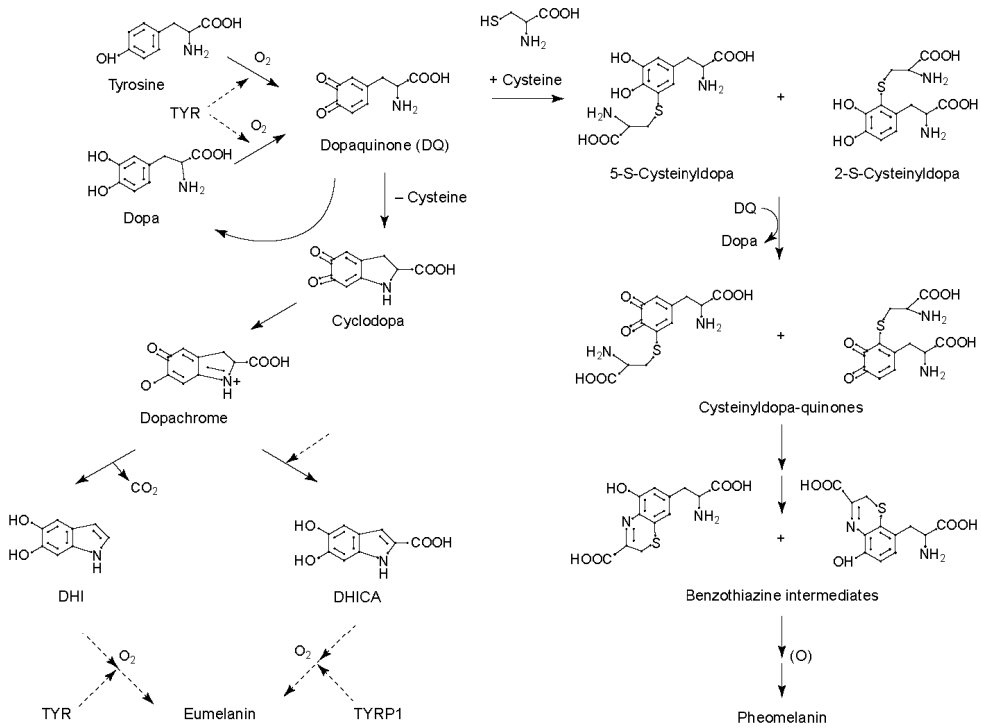


Figure 1: The formation of melanin pigment taken from Sturm *et al.* [19].

melanin known as eumelanin and pheomelanin are produced depending on the presence or absence of the amino acid cysteine [19] as can be seen in the melanin pigment pathway in Figure 1, taken from Sturm *et al.* [19]. Eumelanin is a brown-black form of pigment that is responsible for dark colouration and is packaged in ovoid eumelanosomes, whereas pheomelanin is a red-yellow pigment produced in granular immature pheomelansomes [20]. It is the difference in ratios for the two forms of melanin that produce the visual metamorphoses that can be seen in individuals with variation in eye, hair and skin colour [21].

The distribution of melanin differs in the skin, hair and iris. In the skin, the melanocytes located in the basel layer of the epidermis transfer melanosomes to adjacent keratinocytes that eventually migrate to the upper levels of the epidermis [22], where melanogeneis appears to be continuous [23]. In terms of skin colour there are also differences related to the morphology of the melanocytes and the environment in which melanogenesis takes place [24]. In the hair, the hair bulb is the only site of melanin production. Melanocytes transfer melanosomes to surrounding immature keratinocytes that eventually differentiate and migrate to form the pigmented hair shaft [25]. Melanogeneis only takes place during the anagen phase of the hair growth cycle (hair shaft formation period that lasts 3-5 years) [25]. In contrast to hair and skin, melanosomes are only found in the melanocytes of the iris and the type of melanin and its density and distribution determine eye colour [19]. Differences in pigmentation are due to two major factors, the amount and type of melanin made in the melanocytes (ratio of eumelanin to pheomelanin), and the shape and distribution of the melanosomes [15]. Lightly pigmented skin is enriched in light brown eumelanin and yellow/red

pheomelanins with the melanosomes less pigmented, smaller in size and packaged in groups [26]. Darkly pigmented have more melanin enriched with eumelanin and melanosomes are more pigmented, larger but single units [27]. Figure 2 taken from Barsh *et al* [26] is an illustration of skin colour population differences in the pattern of melanosome size and distribution.

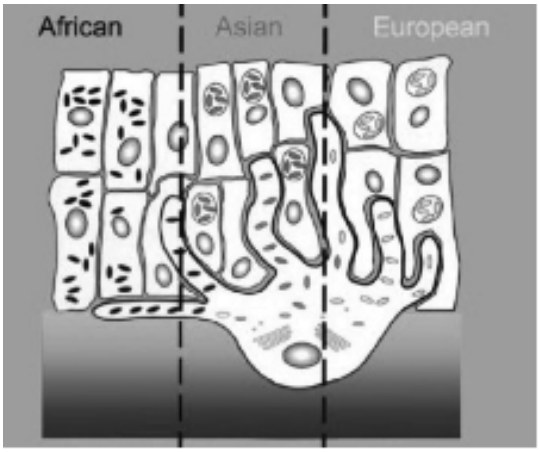


Figure 2: skin colour population differences and melanosome patterns of size and distribution, taken from Barsh *et al.* [26].

In the case of hair colour, individuals with black hair have the highest eumelanin-pheomelanin ratio while those with blond or light brown show intermediate ratios and lastly red hair individuals have the highest pheomelanin levels [22]. This can be seen in a visual example of a hair follicle of dark pigment in Figure 3.

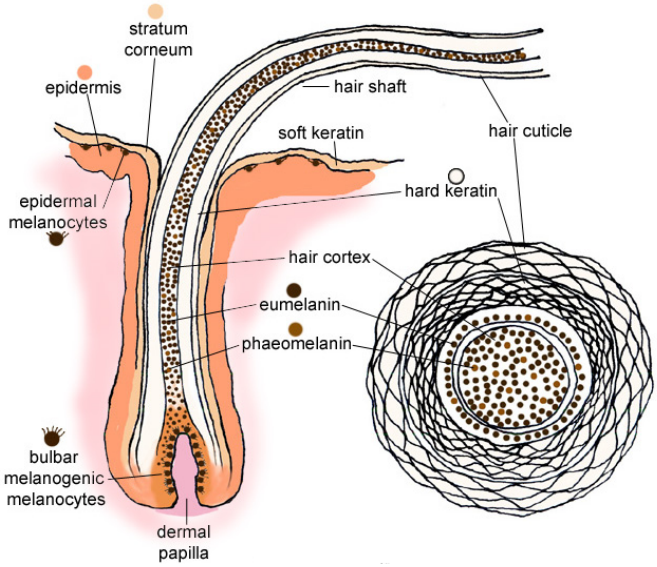


Figure 3: Schematic diagram of a darkly pigmented hair follicle, taken from (www.hennaforhair.com).

For eye colour, brown irises have large amounts of melanin and high numbers of melanosomes that absorb incoming light to reveal a dark colour shade [19]. Although blue eyes have a similar number of melanocyte cells to brown eyes, in contrast, they have low melanin content and few melanosomes, this enables light to penetrate the stroma with some being absorbed in the inner eye while the rest is scattered giving the perceived appearance of blue colour [19]. Green and hazel irises have intermediate amounts of melanin [19]. Figure 4 is a visual representation of the basis of human eye colour taken from Sturm and Frudakis [19].

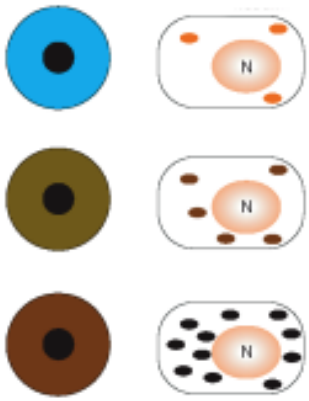


Figure 4: the basis of human eye colour, taken from Sturm and Frudakis [19]. Iridal melanocytes and melanosomes.

From worldwide general knowledge and studies that have categorised modern day skin colour distribution, Figure 5 illustrates skin colour variation worldwide taken from (www.anthro.palomar.edu/adapt/adapt_4.htm), which unifies data collected by the geographer R.Biasutti [28] prior to 1940. It displays a strong correlation with latitude with darker skin colour in equatorial and tropical areas (sub-Saharan Africa, South Asia, Australia and Melanesia) than in areas located far from the equator. Due to this phenomenon, many investigations into the evolution of skin colour have tried to answer why such an obvious trend occurred and if it can be measured on a population basis. The underlying factor seems to be UVR intensity and Joblonski and Chaplin [29] gathered such evidence using skin reflectance measurements in populations throughout the world.

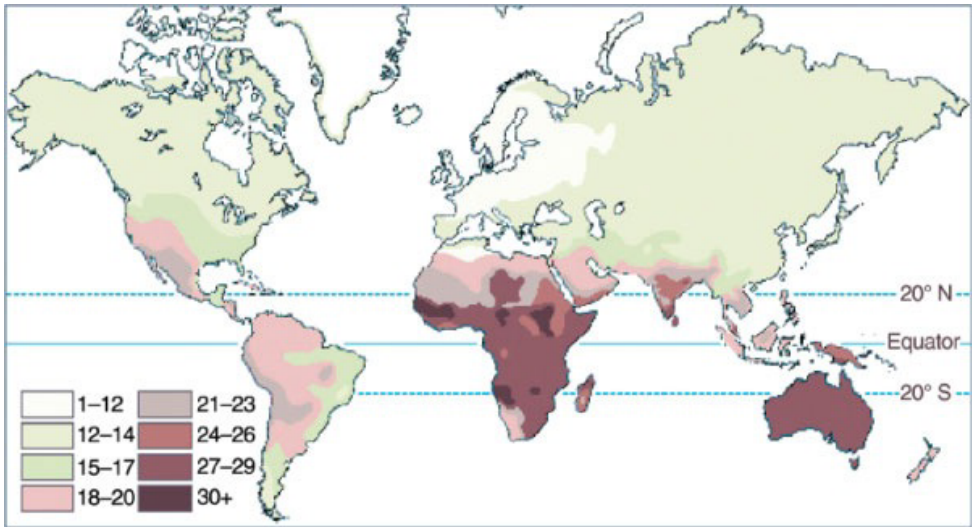


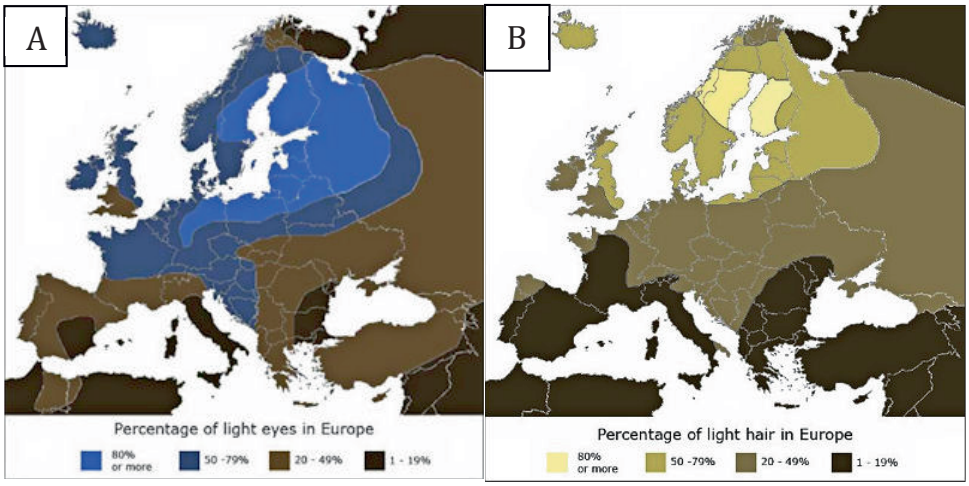
Figure 5: Skin colour variation worldwide, taken from (www.anthro.palomar.edu/adapt/adapt_4.htm)

Many hypotheses have been put forward to explain the evolution of skin pigmentation in human populations, taking melanin levels and latitude

into account facilitating vitamin D synthesis [29, 30] but also the influence of sexual selection in lighter pigmented individuals [9, 31]. Lastly, there has been a lot of scientific interest into when approximately light skin colour first evolved. Recently it's evolution has been estimated to have occurred after the out-of Africa migration in Europeans and East Asians about 30,000 years ago, with more selective European-specific alleles starting much later within the last 11,000 – 19,000 years ago [10].

In contrast to skin pigmentation, variation in eye and hair colour is much more geographically restricted to Europe. The ancestral phenotype in the majority of human populations have dark iris (brown) and dark hair (black) colour. However, eye and hair colour variation are primarily found in European populations with lower frequency in Middle Eastern, North African, West Asian, and South Asian populations [19]. Figure 6A and B are a modern day representation of iris colour and hair colour within Europe, taken from Beals and Hoijer [32] (found in colour at http://www.eupedia.com/europe/maps_of_europe.shtml)

Figure 6: Modern day representation of human iris (A) and hair (B) colour in Europe from (http://www.eupedia.com/europe/maps_of_europe.shtml)



Human eye (iris) and hair colour are highly polymorphic phenotypes in people of European descent including surrounding regions such as the Middle East or Western Asia [32], and are traits under strong genetic control [19]. Brown eye colour and black hair colour are assumed to reflect the ancestral human state [33] and are present everywhere in the world, including Europe in lower frequencies especially in the northern countries [32]. Non-brown eye colours and non-black hair colours are assumed to be of European origin and the increases in frequency as seen today are proposed to have been driven by positive selection during early European history, perhaps a result of colour preferences in human mate choice [9, 34]. Before work on this thesis began, there was no data supported time stamp for the evolution of eye and hair colour variation within Europe. It has only been speculated that variation must have arisen during the great northern migration to the northern part of Europe that took place in the Neolithic periods about 6-10,000 years ago [35]. To explain normal pigmentation variation in modern day individuals, it is essential to look into the genetics behind the trait, associated variants and its prediction using DNA phenotyping methods. This information can also be found by looking at the genetic basis for differences in physical traits between human populations through the use of evolutionary models of adaptation and natural selection [15].

The genetics of human skin, eye and hair colour

In the early days of human pigmentation genetics, it was the use of pigmentation disorders combined with a comparative genomics approach [36, 37], using animal pigmentation models that put forward the first few genes associated with human pigmentation. The most dramatic

examples of gene inactivation when it comes to human pigmentation are the albinism phenotypes of oculocutaneous albinism *OCA1* and *OCA2*, which result in a complete loss of all pigment in iris, hair and skin within an individual. Many loci have been identified over the years in mammalian species through the characterisation of naturally occurring or artificially induced mutations affecting melanin amount which include the genes *TYR, OCA2, TYRP1* and *SLC45A2 (MATP)* [38]. Another important gene that came out of such research on the melanin pathway was the melanocortin coupled receptor gene 1 (*MC1R*). The role of this gene in normal human pigmentation was clarified by studies showing an association of variants of *MC1R* with red hair and fair skin [39-41]. It has a critical role in switching between eumelanin and pheomelanin synthesis in the melanocytes [39]. The agouti protein (*ASIP*) was also shown to act as an inverse agonist of *MC1R* in mouse melanocyte cell culture studies [42], which provides support for its allelic associations with lighter or darker pigmentation phenotypes in humans [43-45]. Another important gene identified through comparative genomics in relation to human pigmentation, was *SLC24A5*, the homologue of the zebrafish golden gene mutation [46], which was implicated in human skin colour through the analysis of its allelic distribution worldwide. Also the *KITLG* gene was implicated in human pigmentation variation by the association of its ancestral allele with skin colour and strong linkage for the derived allele within Europeans and East Asians [11, 47, 48].

As the determinants of human skin, eye, and hair colour fall into the quasi-Mendelian inheritance pattern of a polygenic trait, only a few major genes show high effects with many additional modifier genes [20]. Because of their small effect size, the latter genes are usually difficult to

identify. With the application of genome-wide association studies (GWAS) to pigmentation traits, it is now possible to find more pigmentation genes. GWAS searches through large SNP databases of genotype-phenotype information for genes implicated in specific traits. For example, rather than searching for associated SNPs with pigmentation variation, it treats skin, eye and hair colour as separate phenotypes to find more specific markers. The use of these methods is strictly population-based; therefore GWAS scans must consist of a sole population such as Europeans, to limit the possibility of population stratification seen in significantly associated SNP results. Due to the fact that eye and hair colour pigmentation variation is in fact limited to the European population, there has been much more success with these traits and less so in comparison to skin colour GWAS scans as there is little to no variation within populations worldwide. However only one GWAS scan to date [49] has revealed associated SNPs in relation to skin colour within the European population from the *IRF4* gene. Another GWAS among a South Asian population [50] revealed skin colour associations with variants from three genes, *SLC45A2, TYR* and *SLC24A5*. It was known from a previous study [46], in a study on African Americans that skin colour associated variants were located in the *SLC24A5* gene. Studying skin colour pigmentation effects between populations, Myles *et al.* [51] measured allele frequencies in Europeans, Chinese and Africans for 24 pigmentation genes from two large-scale SNP data sets. The study revealed that the *DCT* gene was strongly associated with pigmentation control in Asians. The *ASIP* and *OCA2* genes appeared to play important roles in European and Asian patterns, while the *SLC45A2 (MATP)* and *TYR* genes affect is seen only in Europeans [51]. In terms of the DNA phenotyping of skin colour, unfortunately there is not enough

research done on the predictive capabilities of variants associated with this trait especially because knowledge of skin colour associated genes has not reached its full potential. This is due to the traits variation existing between different populations and not within a population to a high degree and the difficulty of using current GWAS approaches to find these variants. Therefore DNA-based prediction of skin colour has still a way to go before it becomes a reality.

At present, the majority of studies have been successful in finding new eye and hair colour phenotypes; in particular eye colour is currently the most advanced in terms of the number of genes associated with the trait and research into its DNA-based prediction. In recent years, intensive studies have led to a monumental increase in the genetic understanding of human eye colour, via genome-wide association and linkage analysis or candidate gene studies [33, 34, 43, 45, 49, 52-57]. The *OCA2* gene on chromosome 15 was originally thought to be the most informative human eye colour gene due to its association with the human P protein required for the processing of melanosomal proteins [58], and the fact that mutations in this gene result in pigmentation disorders such as albinism [37]. However, recent studies have shown that genetic variants in the neighbouring *HERC2* gene are more significantly associated with eye colour variation than those in *OCA2* [33, 34, 49, 52, 53]. Also, one of the most significant non-synonymous single nucleotide polymorphisms (SNPs) associated with eye colour, rs1800407 located in exon 12 of the *OCA2* gene, acts only as a penetrance modifier of rs12913832 in *HERC2* and is only to a much lesser extent independently associated with eye colour variation [53]. It was proposed that genetic variation in *HERC2* acts as a functional regulator of adjacent *OCA2* gene activity [33, 34, 53, 59],

and this relationship has been recently proven in a functional genetic study [60]. It shows that the ancestral *HERC2* rs12913832A-allele recruits transcription factors, which, in combination with increased chromatin looping to the nearby promoter of the pigmentation gene *OCA2*, facilitates *OCA2* expression, leading to enhanced melanin production and ultimately dark pigmentation. In contrast, chromatin-loop formation, transcription factor recruitment, and *OCA2* gene expression are all reduced in lightly pigmented melanocytes carrying the derived rs12913832G-allele [60]. While the *HERC2/OCA2* region harbours the most blue and brown eye colour information, there are also other genes identified that contributed to eye colour variation, such as *SLC24A4*, *SLC45A2 (MATP), TYRP1, TYR, ASIP* and *IRF4*, although to a much lesser extent [43, 45, 49, 52, 57].

The genetic basis of human hair colour variation has also been studied quite considerably over the last few years. Valverde *et al*. [39], found that red hair colour is mainly associated with polymorphisms in one single small gene, *MC1R*. This information has also been confirmed in many other studies that have been performed on various population samples [43, 49, 52, 59, 61-64]. A GWAS on individuals from the Icelandic population produced additional data on red hair colour inheritance, which revealed two SNPs in the *ASIP* gene that represent an *MC1R* antagonist and are significantly associated with red hair colour [45]. Using a candidate gene approach, a position in the 3'-UTR of the *ASIP* gene was also found associated with dark hair colour in European populations [43, 65]. This approach also uncovered two non-synonymous SNPs in *SLC45A2* (*MATP*) with association to dark hair colour with several confirmation studies [66-68]. There have been several large GWA studies that have also

found other SNPs with an association to human hair colour variation. One of the earliest GWAS scans for hair colour [45, 52] found variant associations previously implicated in human pigmentation such as *MC1R, HERC2-OCA2* and *KITLG* including the *SLC24A4* gene, a member of the *SLC45A5* family [46]. Also an intergenic region between *IRF4* and *EXOC2* was identified [52]. Another independent GWAS also identified *IRF4* and *SLC24A4* as being linked with hair colour [49], as well as confirming the roles of SNPs within the *HERC2-OCA2, MC1R* and *SLC45A2* loci. Sulem *et al.* [45] also found two coding variants within the *TPCN2* gene which impacts on blond versus brown hair colour. Other associated genes found through these GWAS are *TYR, TYRP1, KIF26A,* and *OBSCN* [45, 49, 52]. Branicki *et al.* [69] also examined *HERC2-OCA2* and not only confirmed the association of *HERC2* rs12913832 with eye colour, but also showed that this SNP is also significantly associated with skin and hair colouration.

The potential of pigmentation-associated DNA variants for eye and hair colour prediction

As the strength of a SNP associated with a trait does not necessarily reflect its individual value for the prediction of that trait, it is an important step to follow-up association results and to assess each and every SNP for its predictive value. For eye colour this was previously done in a systematic study investigating the predictive effect of 37 eye colour associated SNPs from 8 genes in 6168 Dutch Europeans for which eye colour was available [70]. This study demonstrated that using 15 SNPs from eight genes, blue and brown eye colours can be predicted with >90% prevalence-adjusted population based accuracy. This accuracy as expressed by the area under the receiver characteristic operating curve (AUC) found that most eye

colour information is provided by a subset of just six SNPs from six genes [70] (Figure 7).

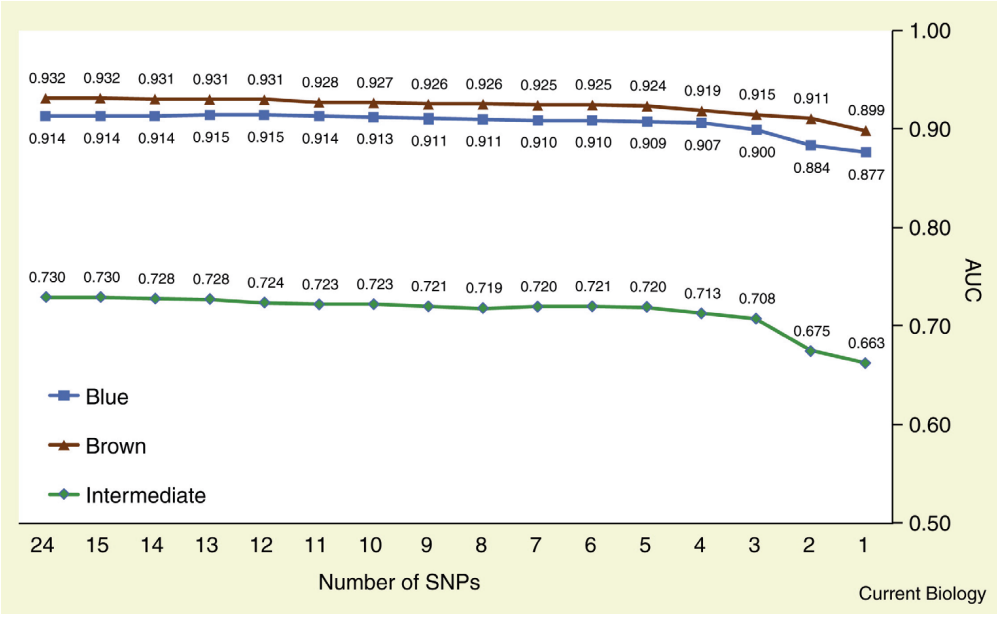


Figure 7: Contribution of 24 SNPs to the prediction accuracy of human eye (iris) color in Dutch Europeans of the Rotterdam Study. Prediction performance measured by AUC for the model based on multinomial logistic regression (Y-axis) was plotted against the number of SNPs included in the model (X-axis). For each step, the lowest contributor in the model building set (n = 3804) was excluded from the model; the model was rebuilt and used to predict eye color in the model-verification set (n = 2364). The additional 13 eye colour associated SNPs also investigated had no independent prediction effect on eye colour. Taken from Liu *et al.* [70]

It is important to understand that the AUC values here represent the models population-based accuracy estimates. It measures the accuracy of eye colour prediction taking into account the whole population for which it was measured. Although this approach does not provide individual-based accuracies for which forensic DNA phenotyping would find its final use, it provides useful information on the suitability of

a SNP, in combination with other SNPs, to predict a trait such as eye colour. In order to predict on an individual level and to provide probability values suggestive for an individualised phenotypic prediction, a prediction model has to be developed that is based on large numbers of genotype and phenotype data. For the final DNA phenotyping application in terms of forensics, this would require a suitable multiplex assay to be developed consisting of these markers, which would allow efficient DNA genotyping of casework samples. In the case of eye colour, this model and assay should consist of the 6 highest ranking predictive SNPs from Liu *et al.* [70]. Such an assay and model, when produced, would then allow the production of probability values for brown, intermediate and blue eye colour of an individual from DNA, with the highest probability value being the predicted phenotype for that individual. For use in a forensic environment, this eye colour DNA phenotyping tool would require full developmental validation according to internationally accepted standards such as those from the Scientific Working Group on DNA Analysis Methods (SWGDAM). Furthermore, the prediction model would require further assessment of the accuracies provided by these prediction probabilities in relation to phenotypic data from other parts of Europe, to measure the overall tool's individual prediction performance accuracy.

Many of the currently known eye colour-associated SNPs with high eye colour prediction values, including four of the high ranking six SNPs in the above study, are located within introns without currently known functional evidence for causal trait involvement. This is apart from the most recent publication on rs12913832 from *HERC2* and its interaction with the *OCA2* gene [60]. Other SNPs most likely provide eye colour information due to physical linkage with causal but currently unknown

variants. This is due to the majority of SNP microarrays, used in genome-wide association studies (GWAS) of complex traits, which include eye colour, being strongly biased towards non-coding markers. It is important to be aware that due to the assumed positive selection history of non-brown eye colour in Europe, it can be expected that these non-causal alleles, with association to non-brown eye colour in people of European (and neighbouring) ancestry, also exist in individuals of different ancestries that do not exhibit non-brown coloured eyes. Therefore it may be possible that these SNPs can cause incorrect prediction outcomes. It is only worldwide data analysis on these prediction SNPs that will give us more information to waive these concerns, and of course the testing of the eye colour prediction tool as a whole to confirm its accuracy.

As elaborated in the previous subchapter, the genetic basis of human hair colour variation has also been studied quite well, with the inheritance of red hair in humans already explained to a significant extent from the *MC1R* gene and its place in the melanin pathway. Due to the fact that *MC1R* SNPs were fairly indicative for red hair, such information has already been implemented in forensic science [71, 72]. However the practical application of red hair colour prediction alone, without the ability to predict other hair colours, limits its use as it strongly depends on the population it is applied to. In fact, in most European populations red hair colour appears in the smallest frequency of all the categorical hair colours, therefore a test only suitable for red hair would have limited practical use. Although research on the genes associated with hair colour have been quite successful, the predictive contribution of highly associated hair colour variants has not yet been measured at the time work on this thesis had begun, apart from the predictive capabilities of

MC1R DNA variants on red hair. Consequently, a similar systematic study as done by Liu *et al.* [70] for eye colour would be an appropriate design to inspect for hair colour prediction accuracies that could be achieved on a population level using known associated hair colour variants. From that knowledge, a prediction model and multiplex genotyping assay for hair colour DNA prediction would then need to be developed. The model should consist of large numbers of genotypes from the most predictive DNA variants, and phenotypes from different parts of Europe to facilitate the highest prediction levels of hair colour variation. The genotyping multiplex assay consisting of the most predictive DNA variants for hair colour would also need to be validated according to international standards.

Aims and outline of this thesis

Building-up on previous knowledge from pigmentation genetics, the work in this thesis aims to develop and test genotyping assays and provide prediction systems designed for the DNA phenotyping of human eye and hair colour in various applications, with a particular focus on forensics. We have concentrated on two of the most obvious externally visible characteristics, eye and hair colour, because previous genetic research had already delivered a large collection of associated DNA variants. Another more forensically motivated reason to focus on eye and hair colour was that these traits are often described in eye-witness reports already used in police investigations. The studies presented here have been conducted using laboratory techniques such as SNP multiplex genotyping, including their design, optimisation and application to large numbers of samples, as

well as statistical approaches used in the prediction of pigmentation traits from DNA variants.

Chapter 2 concentrates on eye colour and consists of four studies related to its prediction in humans. When this thesis work started, there was no tool available that was capable of predicting eye colour from DNA. In **Chapter 2.1,** I describe the design, optimisation and use of such a tool, the IrisPlex system. For every new DNA method used in forensic laboratories, it must pass the required guidelines for implementation and use in casework; therefore in **Chapter 2.2,** I describe a further assessment of the newly developed IrisPlex genotyping assay by performing vigorous tests to ensure its use in accredited forensic laboratories worldwide. To confirm that a statistical model is performing with high levels of accuracy, it is important to assess its reliability on a large number of independent samples. Therefore, in **Chapter 2.3,** I present an evaluation of the IrisPlex models performance on over 3800 individuals from seven sites around Europe. The classification of human eye colour phenotypes is currently at a categorical level; however this description does not represent the true nature of eye colours continuous gradient. Therefore in **Chapter 2.4,** I describe the development and application to DNA data of a new computational approach to estimate continuous eye colour from high-resolution eye imagery. I also show how this new quantitative approach to eye colour phenotyping improves genetic association and DNA prediction. In **Chapter 3,** I present two studies that cover hair colour prediction in humans, in combination with eye colour prediction using knowledge described in Chapter 2. When work on this thesis began, there was no tool available for the DNA prediction of human hair and eye colour. In **Chapter 3.1,** I describe the design, development and use of the

new HIrisPlex system which is not only capable of predicting hair colour from DNA but can also simultaneously predict the eye colour of that individual. Every tool requires developmental validation before it can be used in forensic laboratories on casework samples. Therefore in **Chapter 3.2,** I focus on the assessment of the HirisPlex genotyping assay following strict international guidelines to enable its use in external forensic laboratories worldwide. Using knowledge and tools presented in Chapters 2 and 3, **Chapter 4** consists of two studies that show the application of eye and hair colour DNA prediction for anthropological and evolutionary questions. In **Chapter 4.1,** I report on the investigation of eye and hair colour from available genome sequence data, and newly collected DNA samples of human remains dated to be thousands of years old. The ultimate aim of this chapter is to unveil the time-wise origin of eye and hair colour variation in Europe. In **Chapter 4.2,** I show how robust the HIrisPlex system is at obtaining eye and hair prediction information from DNA extracted from human remains up to hundreds of years old. This includes the eye and hair colour DNA-based prediction of a historical figure that substantiated documented knowledge. Finally, **Chapter 5** provides a general discussion of the findings described in chapters 2-4, as well as an outlook on future research.

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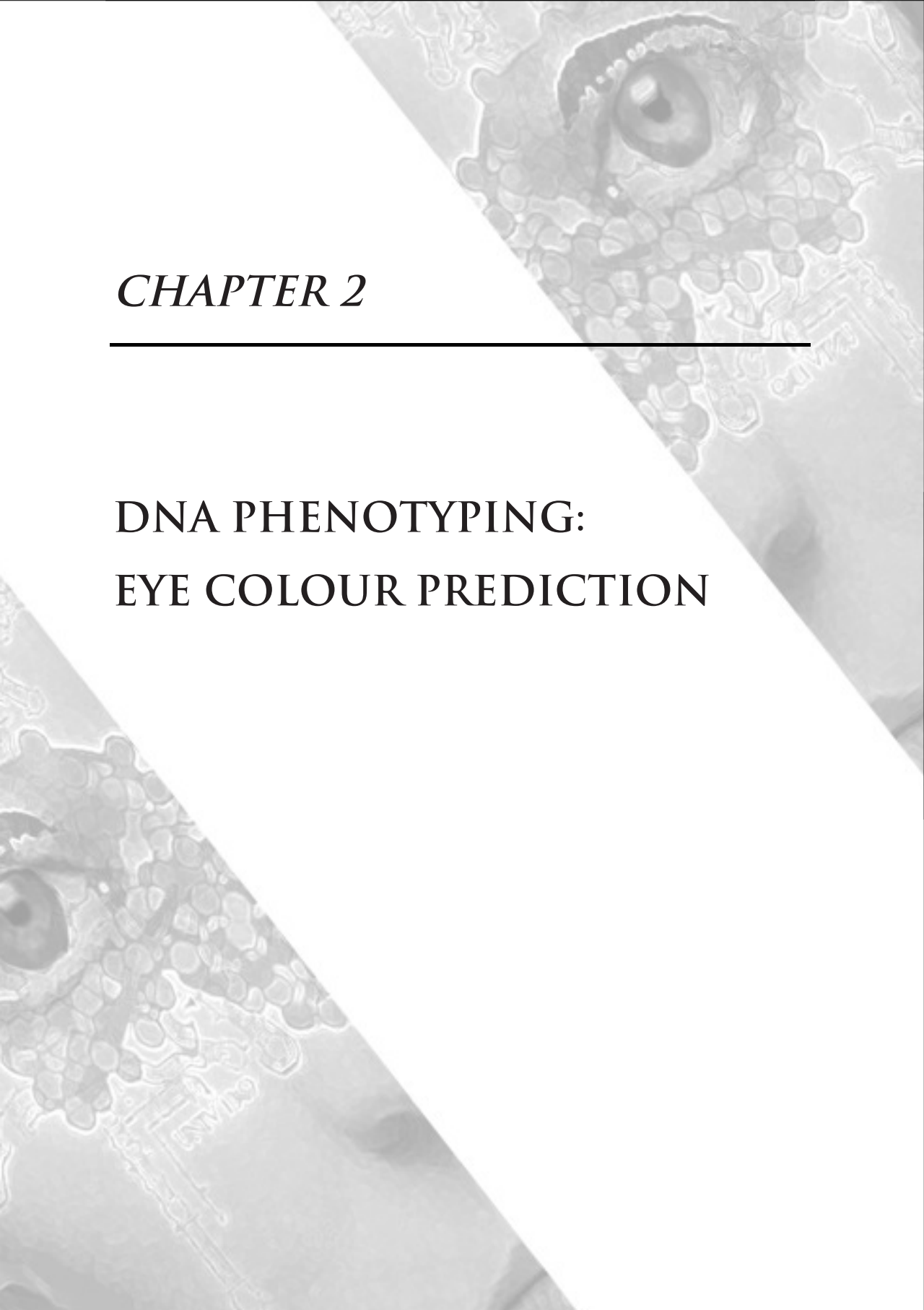

CHAPTER 2

DNA PHENOTYPING: EYE COLOUR PREDICTION

CHAPTER 2.1

IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information.

Forensic Science International: Genetics 5, 170-180 (2011)

ABSTRACT

A new era of 'DNA intelligence' is arriving in forensic biology, due to the ability to now predict externally visible characteristics (EVCs) from trace amounts of biological material. EVC prediction is expected to help concentrate police investigations towards finding unknown suspects in cases where conventional DNA profiling fails to provide informative leads. Here we present a robust and highly sensitive single multiplex system for genotyping the 6 most predictive eye colour SNPs for forensic applications. The system, based on SNaPshot chemistry and capillary electrophoresis widely used in forensic laboratories, displays high levels of accurate blue and brown eye colour prediction from as little as 31 pg of DNA, approximately 6 human cell equivalents. The reproducibility and accuracy of this 6-SNP-eye colour prediction test is demonstrated based on newly generated data of 40 individuals with phenotypic information. We also obtain insights into the worldwide distribution and genetic diversity associated with eye colour variation in Europeans using these 6 SNPs in 51 worldwide populations from the HGDP-CEPH set, which questions the need for prior ancestry testing in eye colour prediction. As this genetic eye colour prediction test is capable of immediate implementation, it represents one step forward in the creation of a fully individualised EVC prediction system for use in forensic DNA intelligence work.

INTRODUCTION

Predicting externally visible characteristics (EVC) using informative molecular markers, such as those from DNA, has started to become a rapidly developing area in forensic genetics [1]. With knowledge gleaned from this type of data, it could be viewed as a 'biological witness' tool in suitable forensic cases, leading to a new era of 'DNA intelligence' (also called forensic DNA phenotyping); an era in which the externally visible traits of an individual may be defined solely from a biological sample left at a crime scene. The most relevant cases for DNA-based EVC prediction would be those in which the evidence sample does not match either a suspect's conventional STR profile or any from a criminal DNA database and also where no additional knowledge about the sample donor exists. DNA-based EVC prediction is also suitable in cases where eye witnesses are available, but their statements about the appearance of an unknown suspect may wish to be confirmed before use in intelligence work. At present, the genetics of individual-specific EVCs remain largely unknown, with little promise for forensic applications. In contrast, the genetic bases of a number of group-specific EVCs such as eye colour are being discovered, and prediction models are expected to be available for forensic genetics in the near future. In certain cases, for example, if the police have no evidence on where / how to find a crime scene sample donor, group-specific EVCs are expected to be useful for tracing unknown suspects by focusing intelligence work on the most likely appearance group to which the individual in question belongs [1].

Human eye (iris) colour is a highly polymorphic phenotype in people of European descent and those from surrounding regions, and is

under strong genetic control [2]. Recent years have yielded intensive studies to increase understanding of the genetic basis of human eye colour, via genome-wide association and linkage analysis or candidate gene studies [3-8]. The *HERC2* gene on chromosome 15 has been identified as containing the most information for blue and brown eye colour, followed by the neighbouring *OCA2* gene [3-7]. The *OCA2* gene was originally thought to be the most informative human eye colour gene due to its association with the human P protein required for the processing of melanosomal proteins [9], and mutations in this gene do result in pigmentation disorders [10]. However, recent studies have shown that genetic variants in *HERC2* are more significantly associated with eye colour variation than those in *OCA2* [3-7]. Also, one of the most significant non-synonymous SNPs associated with eye colour, rs1800407 located in exon 12 of the *OCA2* gene, acts only as a penetrance modifier of rs12913832 in *HERC2* and is, to a lesser extent, independently associated with eye colour variation [6]. It is currently assumed that genetic variation in *HERC2* acts as a functional regulator of adjacent *OCA2* gene activity [3-6], although more work is needed to fully establish the functional relationship between these two genes. While the *HERC2*/*OCA2* region harbours most blue and brown eye colour information, other genes were also identified as contributing to eye colour variation, such as *SLC24A4, SLC45A2 (MATP), TYRP1, TYR, ASIP* and *IRF4,* although to a much lesser degree [3, 7, 8, 11, 12].

Here, we present a robust and highly sensitive genotyping system using the previously established 6 most informative eye colour SNPs [13] for accurate prediction of human brown and blue eye colour in forensic applications. This genetic prediction test employs a single multiplex PCR

followed by a single multiplex single-base extension (SBE) reaction using SNaPshot chemistry and capillary electrophoresis technology, as widely applied by forensic laboratories. In addition, we present a prediction model to correctly classify an individual's eye colour solely based on DNA data and illustrate our eye colour prediction test on 40 individuals from various geographic origins with phenotypic information. At present, there is little knowledge of worldwide genotype frequencies for SNPs with strong eye colour association in Europe. Therefore, we also predicted the eye colour of individuals from 51 populations in the worldwide H952 CEPH set [14, 15], given the known distribution of the blue and brown eye-phenotype, there is evidence to support that additional genetic ancestry testing may not be necessary for correctly interpreting eye colour prediction on a worldwide scale.

MATERIALS & METHODS

Sample collection & iris photography

Buccal swabs were taken from 40 volunteers with informed consent. A photographic image of their iris was taken concurrently with a macro lens, ensuring similar distance and light conditions were used for each photo for normalisation. Information regarding the sex and country of birth for each individual was also collected (Supplementary Table 1). DNA was extracted using the QIAamp DNA Mini kit according to the manufacturer's protocol (Qiagen, Hagen, Germany) and samples were stored at 4 °C. We also obtained the H952 subset of the HGDP-CEPH samples representing individuals from 51 worldwide populations [14, 15]. This subset excludes duplicates, mix-ups and also samples that were genotyped with low quality and therefore analyses consisted of a total of 934 individuals from this set (Supplementary Table 2).

Multiplex design, genotyping and sensitivity testing

Six SNPs; rs12913832, rs1800407, rs12896399, rs16891982, rs1393350 and rs12203592 from the *HERC2*, *OCA2*, *SLC24A4*, *SLC45A2 (MATP)*, *TYR* and *IRF4* genes respectively, were used in this study and marker details are available from Table 1. These 6 SNPs were established recently as carrying the most eye colour information from all currently known genetic markers [13]. The six PCR primer pairs were designed using the free web-based design software Primer3Plus [16] using AutoDimer [18] was used throughout the design. The PCR primer sequences can be found in Table 1.

For the multiplex PCR, a total of 1 µl (0.5 – 2 ng) genomic DNA extract from each individual was amplified in a 12 µl PCR reaction with 1 x PCR buffer, 2.7 mM $MgCl_2$, 200 µM of each dNTP, primer concentrations of 0.416 µM each and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems Inc., Foster City, CA). Thermal cycling for PCR and SNaPshot assays were performed on the gold-plated 96-well GeneAmp® PCR system 9700

(Applied Biosystems). The conditions for multiplex PCR were as follows: (1) 95 °C for 10 min, (2) 33 cycles of 95 °C for 30s and 60 °C for 30s, (3) 5 min at 60 °C.

Both forward and reverse SBE primers were designed for each SNP and the six final primers chosen were based on their suitability for the multiplex and the genotype of the resultant product to allow complete multiplexing. The primer sequences and specifications can be found in Table 1. The design followed a similar

SNP-ID	Prediction rank from [10]	Chr position	Gene	PCR Product (bp)	Forward PCR primer (5'-3') and Reverse PCR primer (5'-3')	Each primer concentration (µM)	Extension Primer (5'-3') with t-tail for length differentiation	Primer direction	Primer length no tail (bp)	Total primer length (bp)	Concentration (µM)	Tm (°C)	Alleles detected
rs12913832	1	15–26039213	*HERC2*	87	TGGCTCTCTGTGTCTGATCC GGCCCCTGATGATGATAGC	0.416	ttttttttttttttttttttt- GCGTGCAGAACTTGACA	Reverse	17	41	0.2	55.0	T/C
rs1800407	2	15–25903913	*OCA2*	127	TGAAAGGCTGCCTCTGTTCT CGATGAGACAGAGCATGATGA	0.416	ttttttttCCCACACCCGTCCC	Reverse	14	23	1.0	57.3	C/T
rs12896399	3	14–91843416	*SLC24A4*	104	CTGGCGATCCAATTCTTTGT CTTAGCCCTCGGGTCTTCATG	0.416	ttttttttttttttttttttttta- TCTTTAGGTCAGTATATTTTGGG	Forward	23	53	0.15	54.5	G/T
rs16891982	4	5–33987450	*SLC45A2 (MATP)*	128	TCCAAGTTGTGCTAGACCAGA CGAAACAGGAGTCGAGGTTG	0.416	ttttttttttAAACACGGAGTTGATGCA	Forward	18	29	0.22	55.9	C/G
rs1393350	5	11–88650694	*TYR*	80	TTCCTCAGTCCCTTCTCTGC CGGAAGGTGAATGATAACACG	0.416	ttttttttttttttttttta- TTTGTAAAAGACCACACAGATTT	Reverse	23	47	0.1	55.6	T/C
rs12203592	6	6–341321	*IRF4*	115	ACAGGGCAGCTGATCTCTTC GCTAAACCTGGCACCAAAAG	0.416	ttttttttttttt- AAAGTACCACAGGGGAATTT	Reverse	20	35	0.3	55.2	G/A

Table 1: SNP markers included in the single multiplex test system for eye colour prediction ordered according to prediction rank, their molecular details, and genotyping information.

protocol to the PCR primer design ensuring temperatures of approximately 60 °C for the SBE reaction and all possible primer interactions were screened. To ensure complete separation between the products, poly-T tails of varying sizes were added to the six SBE primers.

Following PCR product purification to remove unincorporated primers and dNTPs, the multiplex SBE assay was performed using 1 µl of product with 1 µl SNaPshot reaction mix in a total reaction volume of 5 µl. The following thermocycling programme was used: 96 °C for 2 min and 25 cycles of 96 °C for 10s, 50 °C for 5s and 60 °C for 30s. Excess fluorescently labelled dNTPs were inactivated and 1 µl of cleaned multiplex extension products were then run on an ABI 3130*xl* Genetic Analyser (Applied Biosystems) following the ABI Prism® SNaPshot kit standard protocol (Applied Biosystems). Allele calling was performed using GeneMapper v. 3.7 software (Applied Biosystems). A custom designed bin set was implemented to allow automation of genotyping.

A threshold of 50 rfu for peak intensities was adopted for sensitivity testing (Figure 1) to ensure accuracy of genotyping. Samples from three different individuals (brown, intermediate and blue eye colour) were measured and quantified in a dilution series using the Quantifiler™ Human DNA Quantification kit (Applied Biosystems). Template concentrations from 0.5 ng/µl - 0.015 ng/µl were also run in duplicate to test the overall sensitivity of the multiplex.

Statistical analysis

The formula used in this study for eye colour prediction has been previously published by Liu *et al*. [13]. It is based on a multinomial logistic

regression model. The probabilities of each individual being brown (π_1), blue (π_2), and otherwise (π_3) were calculated based on the sample genotypes,

$$\pi_1 = \frac{\exp(\alpha_1 + \sum \beta(\pi_1)_k x_k)}{1 + \exp(\alpha_1 + \sum \beta(\pi_1)_k x_k) + \exp(\alpha_2 + \sum \beta(\pi_2)_k x_k)}$$

$$\pi_2 = \frac{\exp(\alpha_2 + \sum \beta(\pi_2)_k x_k)}{1 + \exp(\alpha_1 + \sum \beta(\pi_1)_k x_k) + \exp(\alpha_2 + \sum \beta(\pi_2)_k x_k)}$$, and

$$\pi_3 = 1 - \pi_1 - \pi_2.$$

where x_k is the number of minor alleles of the k^{th} SNP. The model parameters, alpha and beta can be found in Supplementary Table 3. These probabilities can be calculated using the macro provided in the supplementary material (Supplementary Table 4). Each individual is classified as being brown, blue or intermediate based on the predicted probabilities derived from the above formula. For example, a phenotypic brown-eyed individual can give a probability value of 0.76 for brown, 0.09 for blue and 0.15 for intermediate.

For the worldwide distribution, a threshold of 0.7 predicted eye colour probability was used for categorisation. For example, an individual is predicted as brown if $\pi_3 > 0.7$, otherwise they are predicted as undefined. This cut off was chosen based on the receiver operating characteristic (ROC) curve derived from the Dutch study [13], where after the false positive rate of 0.3 (corresponding to specificity of 0.7), the decrease of the true positive rate becomes costly, with the possibility of errors increasing, as seen in Supplementary Figure 1 (see below for a discussion on the selection of the appropriate threshold). To evaluate the prediction accuracy on the worldwide samples, we assumed that all

individuals outside of Europe and Western Russia are brown eyed, as phenotypic data are not available for the HGDP-CEPH individuals.

We used the MapViewer 7 (Golden Software, Inc., Golden, CO, USA) package to plot the distribution of SNP genotypes and the predicted eye colour, on the world map. A non-metric multi-dimensional scaling (MDS) plot was also produced to illustrate the pair-wise Fst distances [19] of the 6 eye colour SNPs between populations, using SPSS 15.0.1 for Windows (SPSS Inc., Chicago, USA).

RESULTS & DISCUSSION

Assay design & sensitivity

The design of the multiplex PCR considered amplicon lengths of only 80 to 128 bp, allowing future application to forensic samples that often contain fragmented DNA due to degradation. The SBE multiplex was designed so that extension products were evenly separated by 6 bp in the region of 30–65 bp in length to ensure unequivocal marker differentiation. PCR and SBE multiplex optimizations aimed to balance all SNP alleles, generating similar peak intensities to ensure genotyping accuracy in a wide range of DNA quantities. However, despite extensive efforts, allele balance was not completely achieved e.g. allele T of rs12896399 in its heterozygote state, or allele C of rs16891982 in its homozygote state were lower in comparison (Figure 1). However, this slight imbalance does not affect the genotyping accuracy, unless the DNA quantity falls below the sensitivity threshold, and thus appeared sufficient for practical applications. The assay works optimally between 0.25-0.5 ng of template DNA, but also reveals complete profiles down to a level of 31 pg, or approximately 6 human cell equivalents. Only at 15 pg of DNA template were allelic drop-outs observed for some of the SNPs (Figure 1). Notably, the sensitivity achieved is considerably higher than those previously reported for autosomal SNP multiplexes for human identification purposes (which may be influenced by SNP numbers included). For example, 500 pg of DNA was required for a full profile of 52 SNPs analysed in two SBE multiplexes after a single multiplex PCR [20], and also for a full 20 SNP (plus amelogenin) profile from a single tube PCR reaction [21]. The sensitivity of our SNP multiplex is also considerably higher than that of the commercially available AmpF*l*STR Minifiler kit (Applied Biosystems)

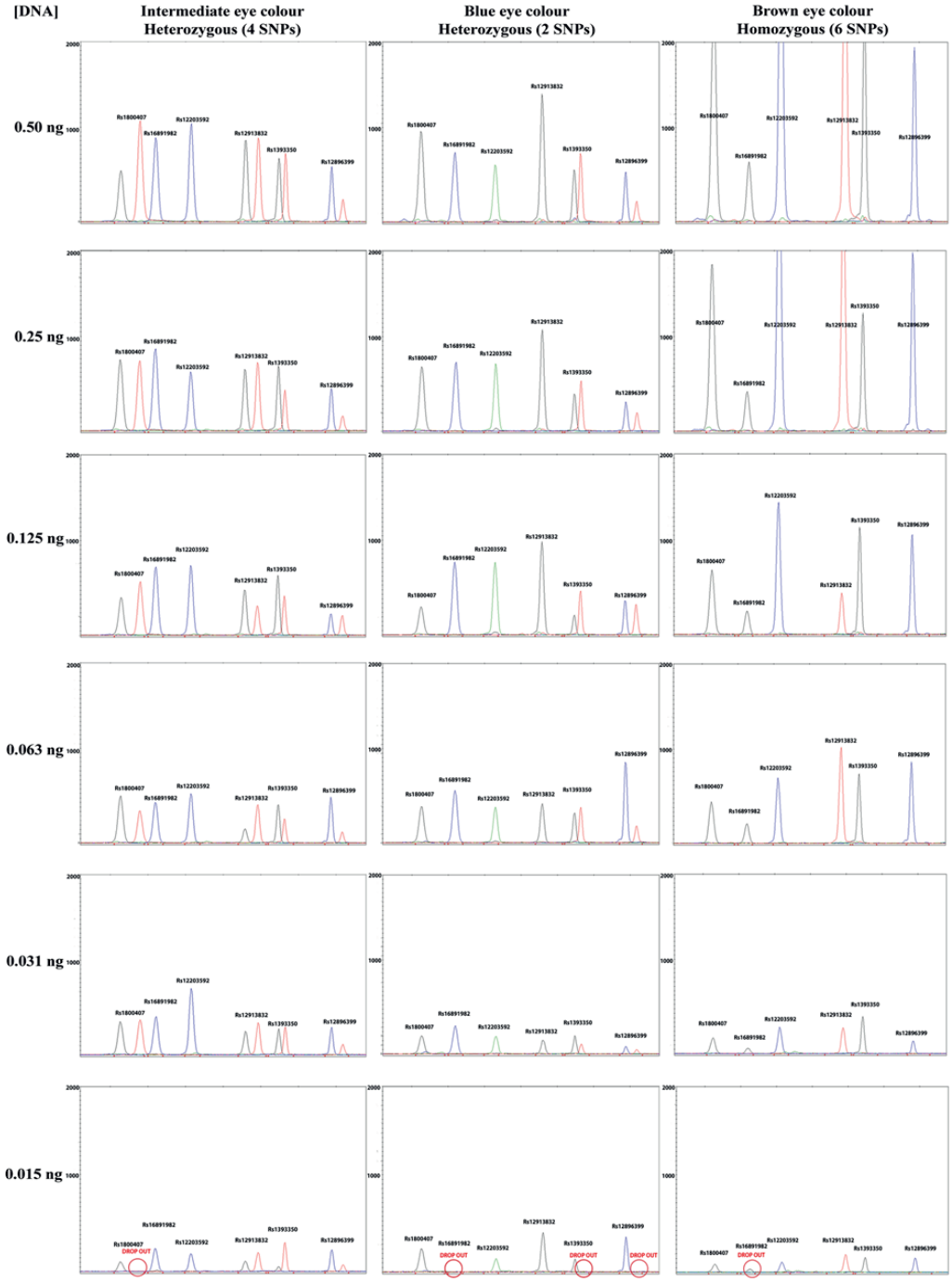

Figure 1. Sensitivity testing of the eye colour multiplex assay. Multiplex single base extension (SBE) products from starting DNA amounts between 0.5 ng - 15 pg used in the prior single multiplex PCR are shown for three individuals with intermediate, blue and brown eye colour phenotypes and confirmatory DNA-based eye colour prediction results. Allelic drop-outs are designated by red circles.

recommended for degraded DNA typing, which requires at least 125pg input DNA for full profiles of 8 autosomal STRs (plus amelogenin) [22]. We therefore expect the sensitivity of our eye colour prediction test to meet the requirements of routine forensic case work in most cases, with it expected to be more successful than multiplex systems currently used in forensic applications.

Prediction probability accuracy

We previously established that the 6 SNPs included in this multiplex assay carry the most eye colour prediction information when analysing all 37 known eye colour associated SNPs in 6168 Dutch Europeans with the 6 SNPs from the multiplex assay alone reaching AUC values of 0.925 for brown and 0.910 for blue [13]. To further illustrate the predictive performance of the multiplex we generated additional 6 SNP multiplex genotypes for 40 individuals from various geographic origins (Supplementary Table 1). Figure 2 represents how the prediction strength is correlated with each individual's eye colour phenotype in terms of the highest probability value, from blue to brown. The images were ordered based on prediction probabilities for blue starting in the upper left corner and for brown starting in the lower right corner of the picture, with the prediction probabilities for all three eye colour categories provided for each sample and eye image. It is evident that there is a clear correlation between the predicted values and the visual representation of the eye colour phenotypes viewed from top to bottom, left to right, thus confirming the accuracy of the 6 SNP prediction model. For 37 (92.5%) of the individuals the genetic eye colour prediction perfectly agreed with the

Figure 2. Eye colour images of 40 individuals of various geographic origins and their estimated eye colour prediction probabilities for blue (Bl), intermediate (Int), and brown (Br) respectively, from applying the 6 SNPs multiplex test. The highest of the three prediction probabilities per individual is highlighted in bold. Eye color images are ordered based on maximal prediction probabilities for blue starting from the upper left corner and for brown starting from the lower right corner. The area in red marks 3 cases with incorrect or non-conclusive eye color prediction results based on the genetic test applied.

eye colour phenotype from visual inspection. Only three individuals (see red box in Figure 2), were incorrectly categorised into their brown/blue categories by the prediction model, or were inconclusive. From this 40 person data set, the correct call rate (sensitivity) of the model when using an accuracy of above 0.7 was 56% for brown eye colour categorisation and 91.6% for blue. However, upon using a 0.5 eye colour probability, 87.5% for brown eye colour categorisation and 91.6% for blue was achieved. The 0.5 probability level successfully illustrates the sensitivity of the model in comparison with sensitivity values of 88.4% for brown and 93.4% for blue eye colour characterisation [13].

The probability level can be altered to achieve higher specificity levels, although this will affect the overall sensitivity of the model. For example, probability levels of 0.9 and above will increase the specificity dramatically for true blue and true brown homozygotes, with 24 out of the 40 individuals showing 100% prediction accuracy. However, using such a high threshold, light and dark intermediates that could be visually viewed as slight variations of blue or brown respectively would then fail to be categorised into blue or brown. These "intermediates" are currently more challenging to define using the present prediction model. Notably, in our previous study involving several thousand Dutch Europeans, we observed that the at the 0.5 threshold, the prediction accuracy for intermediate (i.e. non-blue / non-brown) eye colours was considerably lower at only 0.73 than that seen for blue and brown colours at > 0.91 [13]. We hypothesised that the lower prediction accuracy reached for these intermediate colours may be explained by imprecise phenotype categorisation or the result of unidentified genetic determinants [13]. Hence, more work is needed to find genetic variants with predictive value for the non-blue and non-

brown eye colours. Finally, discrepancies between genetically predicted and true phenotypic eye colour may be caused by the fact that eye colour can change over one's lifetime. However, as this is a rare phenomenon [23], it is not expected to affect our prediction test significantly, but may be a contributing factor as to why we could not assign three test individuals in this study correctly, as well as deviations from 100% prediction accuracy in our previous study [13].

Each SNP included in the multiplex provides mounting genetic information towards the overall prediction accuracy achievable with this 6 SNP multiplex test system, although with different input. As previously established [13], rs12913832 in the *HERC2* gene alone carries most of the eye colour predictive information with an AUC of 0.899 for brown and 0.877 for blue achieved with this single SNP. This is in line with previous association studies showing that this SNP is the most strongly eye colour associated SNP currently known [4, 6, 13]. The additional 5 SNPs from the *OCA2*, *SLC24A4*, *SLC45A2 (MATP)*, *TYR* and *IRF4* genes included in the multiplex assay slightly increase the prediction accuracy as reflected in the prediction rank established before [13] indicating their lower (but still significant) eye colour association in previous studies [7, 8, 13]. Notably, two SNPs, rs1393350 from the *TYR* gene and rs12896399 from the *SLC24A4* gene, reached much lower P-values for association when comparing individuals with blue versus green eyes relative to blue versus brown eyes previously [3]. This may indicate that they contribute more to the blue and intermediate prediction and less to the brown prediction. The P-values and adjusted rs12913832 beta values for the 6 SNPs involved in this prediction model can be found in the supplementary material by Liu *et al*. [13] as the highest ranking SNPs. In general, to understand the impact of

each SNP on the prediction model, two scenarios have been displayed in Figure 3 for the genetic variation in eye colour based on the 6 SNPs presented.

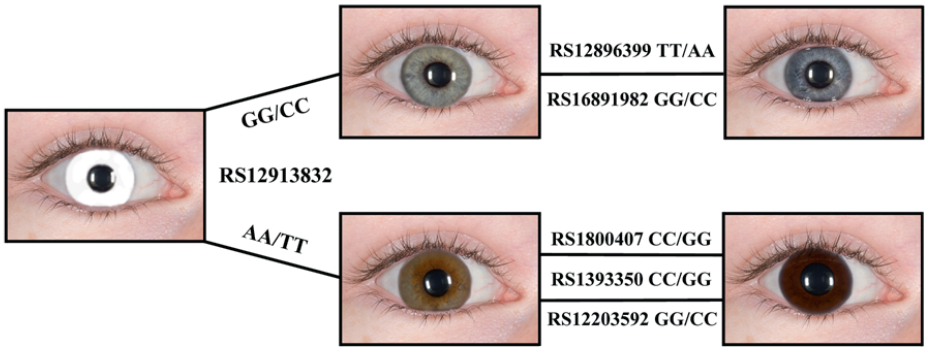

Figure 3. Theorised scenarios for brown and blue eye colour prediction showing the impact of the most influential SNP genotypes from the 6-SNP model.

Worldwide reliability of the prediction test

Non-brown eye colours in humans are basically restricted to people descending from Europe and neighbouring regions, with an increase in frequency from northern to southern Europeans [24], whereas people originating from the rest of the world have brown eye colour, assumed to reflect the ancestral state of modern humans [3]. Non-brown eye colours are assumed to be of European origin, going back to an initial event during the Neolithic expansion (6,000-10,000) [4] and may have been driven by positive selection starting in early European history, perhaps as a result of rare colour preferences in human mate choice [25]. Of the 6 SNPs included in the multiplex assay only two (rs16891982 in *SLC45A2* and rs1800407 in *OCA2*) are located in coding sequences (exons) and represent non-synonymous changes, and for only one (rs12913832 in *HERC2*) a regulatory function is assumed [4]. There is no current

evidence that the remaining three SNPs are causal for eye colour variation in a direct way. In principle it can be expected that genetic variants that are not causal themselves but are associated with a trait may also occur independently of the trait just by chance. This may be particularly true for non-brown eye associated alleles outside of Europe and neighbouring regions; therefore it is of importance to establish the frequency of SNP alleles associated with non-brown eye colours not only in Europeans but also in worldwide populations. If such alleles are found outside of Europe, or in neighbouring regions in people with brown eyes, then a genetic ancestry test would be required to verify the European genetic origin of a sample donor before a non-brown eye colour prediction can be interpreted accurately. We address this issue by investigating the distribution of the 6 SNPs on a worldwide set of individuals.

Figure 4 displays the genotypes of 934 individuals from 51 populations per SNP. In Figure 4(a) there is a clear division for the highest-ranking SNP, rs12913832 where the majority of the blue associated genotype (GG/CC) is confined to Europe and surrounding areas, and rare elsewhere, although this can be expected due to the theory that non-brown eye colour originated in Europe [4]. The blue associated allele of rs16891982 (Figure 4(d)) also displays a confinement to Europe and surrounding regions as far as the Middle East, an area known for its admixture properties [26]. Rs1800407 (Figure 4(b)), which is postulated to act as a penetrance modifier on rs12913832, is less defined, displaying the heterozygous genotype to a higher degree within Europe, as do Rs1393350 (Figure 4(e)) and rs12203592 (Figure 4(f)). Apart from rs12896399 (figure 4(c)), which displays no recognisable trend, there is an increase in frequency of the heterogenic genotypes towards Europe,

which corroborates the high degree of eye colour variation in that region. The presence of non-brown associated genotypes located in regions outside Europe does however seem to increase in the lower ranked SNPs and this can be explained by their lower eye colour association values, although there is not a complete correlation.

The variation in worldwide SNP distributions worldwide underlines the importance of using a combined SNP model to accurately predict eye colour. Figure 5 is an illustration of the predicted eye colours of the CEPH worldwide panel using this model. The eye colour data predicted in this figure also displays a similar trend and percentage of blue and brown eye colour to the study conducted by Liu *et al*. [13] and European phenotypic information recorded in another study [24], thus establishing the accuracy of the prediction model within Europe. Therefore, as brown eye colour is believed to be the ancestral phenotypic state [5], to analyse the data on a worldwide scale (outside of Europe) without precise phenotypic knowledge using these eye colour informative SNPs associated with a European population, we must first look at how the brown eye colour predictions are distributed. Using this model, brown eye colour in the HGDP samples from outside Europe, Middle East and West Asia, i.e. in regions where only brown eyes are expected by anecdotal evidence, revealed an overall brown eye colour prediction of 99.3%. Predicting brown eye colour in HGDP Europeans, Middle Easterners and West Asians, i.e. in regions were non-brown eyes may be expected, revealed a value of 77.4% and finally in HGDP Europeans (including Western Russia) alone, where the highest frequencies of non-brown eyes exist, revealed 45% predicted brown eye colour.

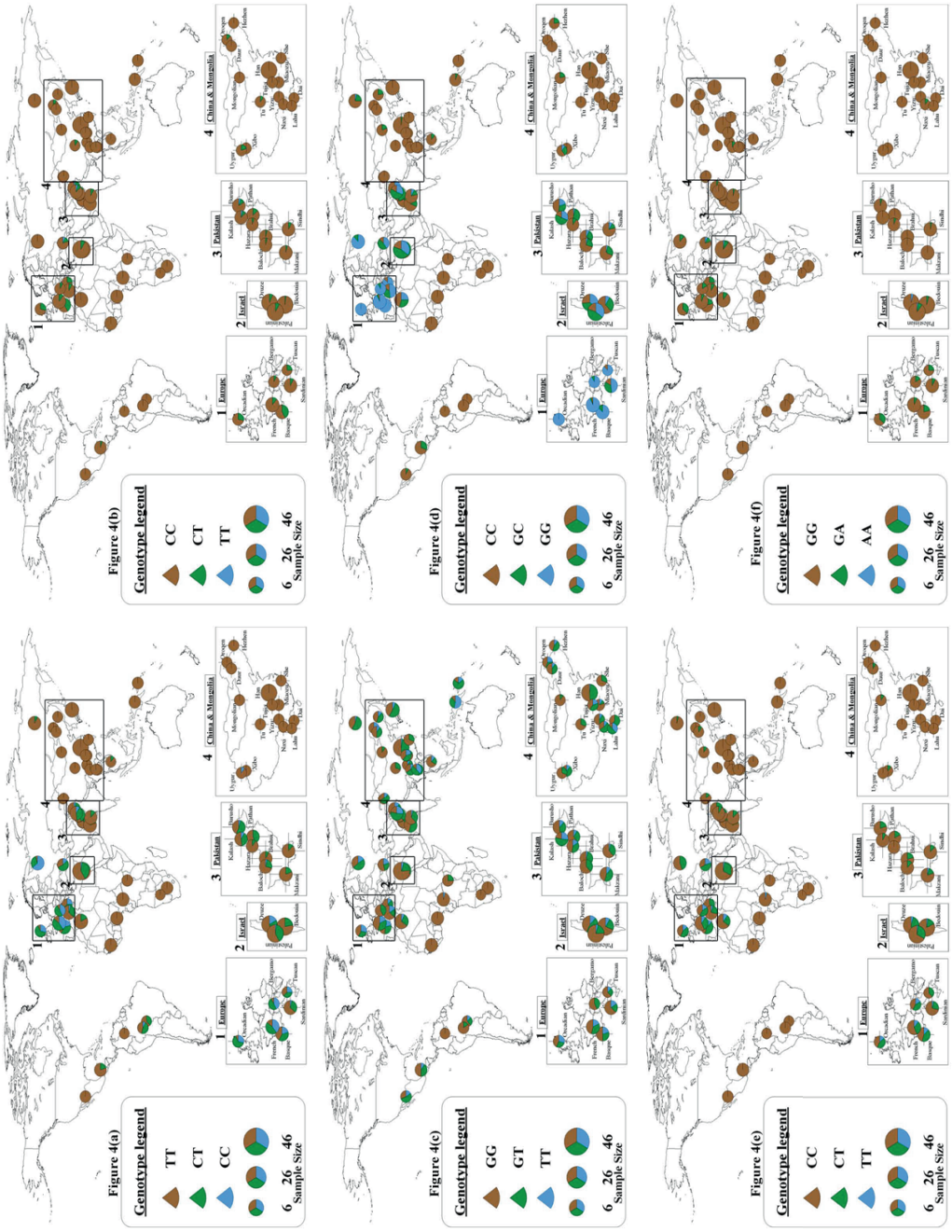

Figure 4. Worldwide genotype distribution of the 6 SNPs that compile the eye colour model from 934 individuals of the H952 HGDP-CEPH set, in order of rank, rs12913832 (a), rs1800407 (b), rs12896399 (c), rs16891982 (d), rs1393350 (e), rs12203592 (f).

Figure 5. Worldwide prediction of eye colour on 934 individuals from 51 populations at a threshold of 0.7 probability categorisation, blue equals a blue predicted phenotype above 70% probability, brown equals a brown predicted phenotype above 70% probability, black equals undefined (less than 70% prediction probability).

Additionally, as demonstrated in a recent study on a Japanese population, where the eye colour phenotype was brown in all individuals, further evidence supports the significant SNP for blue/brown eye colour prediction, as it was only observed in a mono-allelic distribution of the AA/TT genotype [27] without the blue genotype GG/CC being observed in the subset. To further support the accuracy of the prediction model outside of Europe, all the Japanese CEPH samples were predicted accurately as 100% brown. For additional confirmation on the use of this model on a worldwide scale, 100% of the individuals sampled from the 40-person dataset with phenotypic images and whose country of origin is outside of Europe displayed their correct eye colour prediction.

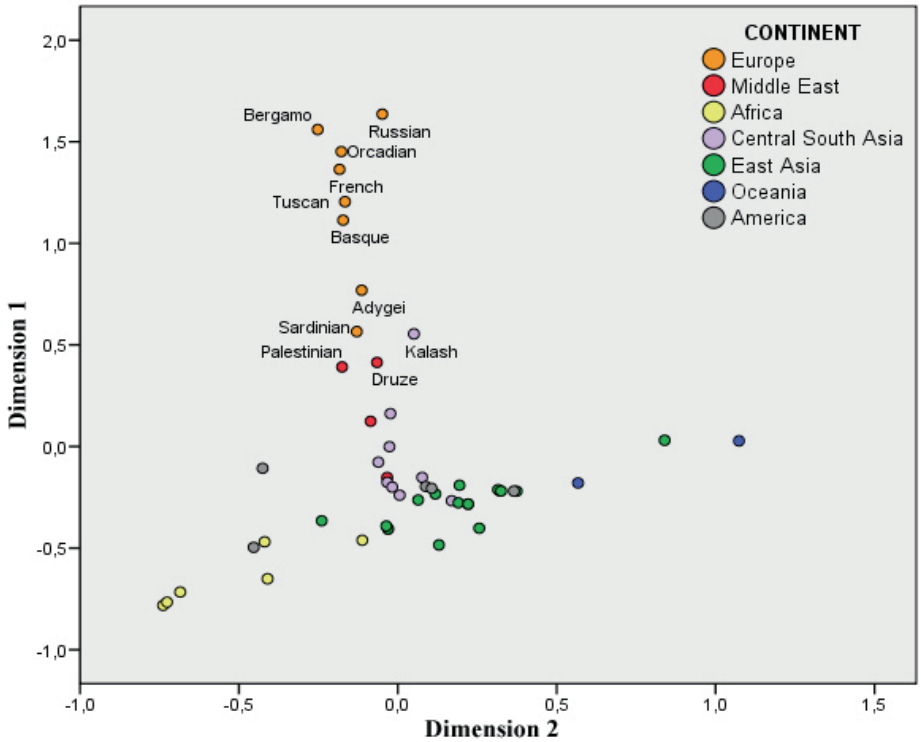

Figure 6.
Multidimensional scaling (MDS) plot of the genetic pairwise distances (Fst) between populations using the 6 SNPs from the eye colour prediction model, grouped into colours by continent/region.

Pairwise Fst values were also calculated from the 6 SNP genetic data and a non-metric multidimensional scale (MDS) plot was produced (stress value 0.2096) (Figure 6). The genetic distance in terms of the 6 eye colour SNPs is representative of the increase in eye colour variation (brown to blue) that exists in Europeans and albeit less so in several groups from the Middle East (Israel) and from Central South Asia (Pakistan) but is absent from all other populations and therefore displays quite a clear separation of Europe from the rest of the world due to this.

Conclusions

We have introduced a single multiplex genotyping system for the 6 currently known most informative eye colour SNPs, which aims to reliably predict blue and brown eye colours with high levels of accuracy when combined in a prediction model. The test assay is extremely sensitive allowing successful analyses of very small amounts of DNA, its design caters for degraded DNA, and it is based on a genotyping technology that relies on equipment widely used by the forensic community. We therefore expect this DNA-based eye colour prediction test will be highly suitable for application to forensic casework, including those with limited DNA quantity and quality. Although the precise phenotype is not known for the worldwide HGDP CEPH samples, the worldwide prediction distribution shows supporting evidence that correct interpretation of the prediction outcomes from this test does not require additional genetic ancestry testing. However without precise phenotypes, the existence of non-brown eye colour outside Europe that cannot be detected by these SNPs may be possible, but highly unlikely as

comparative frequency results obtained by Liu *et al*. [13] and observed phenotypic percentages of brown and non-brown individuals [24] are also reproduced in this study within Europe. The accuracy of prediction therefore, is not believed to differ if this model is used worldwide. However, even considering this supporting evidence, it would be of interest to do a worldwide study on eye colour prediction where phenotypic data is available. As phenotypic trait prediction can create many new avenues of investigation combined with other means of intelligence, the DNA eye colour prediction test presented here is expected to become of great benefit to the forensic community in the coming years.

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Supplementary Material can be found at

http://www.sciencedirect.com/science/article/pii/S1872497310000323

Or the **APPENDICES** of this Thesis.

CHAPTER 2.2

Developmental validation of the IrisPlex system: Determination of blue and brown iris colour for forensic intelligence.

Forenic Science International: Genetics 5, 464-471 (2011

ABSTRACT

The IrisPlex system consists of a highly sensitive multiplex genotyping assay together with a statistical prediction model, providing users with the ability to predict blue and brown human eye colour from DNA samples with over 90% precision. This 'DNA intelligence' system is expected to aid police investigations by providing phenotypic information on unknown individuals when conventional DNA profiling is not informative. Falling within the new area of forensic DNA phenotyping, this paper describes the developmental validation of the IrisPlex assay following the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines for the application of DNA-based eye colour prediction to forensic casework. The IrisPlex assay produces complete SNP genotypes with only 31 pg of DNA, approximately six human diploid cell equivalents, and is therefore more sensitive than commercial STR kits currently used in forensics. Species testing also revealed human and primate specificity for a complete SNP profile. The assay is capable of producing accurate results from simulated casework samples such as blood, semen, saliva, hair, and trace DNA samples, including extremely low quantity samples. Due to its design, it can also produce full profiles with highly degraded samples often found in forensic casework. Concordance testing between three independent laboratories displayed reproducible results of consistent levels on varying types of simulated casework samples. With such high levels of sensitivity, specificity, consistency and reliability, this genotyping assay, as a core part of the IrisPlex system, operates in accordance with SWGDAM guidelines. Furthermore, as we demonstrated previously, the IrisPlex eye colour prediction system provides reliable results without the need for

knowledge on the bio-geographic ancestry of the sample donor. Hence, the IrisPlex system, with its model-based prediction probability estimation of blue and brown human eye colour, represents a useful tool for immediate application in accredited forensic laboratories, to be used for forensic intelligence in tracing unknown individuals from crime scene samples.

INTRODUCTION

In current forensic practice, short tandem repeat (STR) DNA typing is the chosen method for identifying the donor of a biological deposit found at a crime scene. However, if this unknown STR profile does not match a known suspects profile or any from a criminal DNA database, then progress in the case may be halted if no other evidence is present. In cases such as this, the investigation can turn towards alternative DNA intelligence, and predicting externally visible characteristics (EVCs) using informative DNA markers, a new field in forensic genetics described as forensic DNA phenotyping, has started to become a new and exciting area of investigation [1]. At present, although it is not possible yet to predict individual specific EVCs from biological material, group specific EVCs are now achievable. Models for predicting phenotypes such as eye colour from genotypes have been created [2, 3], as well as association likelihood ratios [4]. This DNA intelligence information may be used as a 'biological witness' in multiple circumstances from routine casework to disaster victim and missing person identification if conventional identification procedures fail. The IrisPlex system was developed for such a reason [5]. This assay consists of a single multiplex genotyping system for the six most eye colour informative SNPs according to current knowledge, in conjunction with a statistical prediction model [2] and can correctly predict human blue and brown eye colour with >90% precision. We have previously introduced the method [5]; here we present the developmental validation of this assay following the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines [6]. These guidelines allow an assessment of the quality and limitations of the assay under differing conditions, testing the sensitivity, reproducibility and concordance. It also

tests nonhuman amplification, mixtures, degraded DNA and simulated casework samples and is a necessary step towards the implementation of the IrisPlex system for use on casework investigations in certified forensic laboratories. Through these studies, we provide information on its advantages and limitations, and demonstrate a range of circumstances under which this assay can be used.

MATERIALS & METHODS

A range of human body fluid and tissue samples were obtained from donors with known eye colour phenotypes under informed consent. Test samples included single and multiple source samples, non-human and simulated casework samples (blood, saliva, semen, hair, touched items; see Supplementary Table 1). Duplicate reactions were performed on all samples unless otherwise stated. DNA was extracted from all samples using the QIAamp DNA Mini kit (Qiagen, Hagen, Germany) according to the manufacturer's guidelines and quantified using the Quantifiler Human DNA Quantification Kit (Applied Biosystems Inc., Foster City, USA).

Multiplex design & protocol

The IrisPlex consists of 6 SNPs, rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs12896399 (*SLC24A4*), rs16891982 (*SLC45A2* (*MATP*)), rs1393350 (*TYR*)

SNP-ID	Prediction rank	Chr position	Gene	PCR product (bp)	Forward PCR primer (5'–3') and reverse PCR primer (5'–3')	Each primer conc. (µM)	Extension primer (5'–3') with t-tail for length differentiation	Primer direction	Primer length no tail (bp)	Total primer length (bp)	Conc. (µM)	Tm (°C)	Alleles detected
rs12913832 (1F and 1R)	1	15 – 26039213	*HERC2*	87	TGGCTCTCTGTCTCTGATCC GGCCCCTGATGATGATAGC	0.416	ttttttttttttttttttttttttGCGTGCAGAAC TTGACA	Reverse	17	41	0.2	55	T/C
rs1800407[a] (2F and 2R)	2	15 – 25903913	*OCA2*	127	TGAAAGCCTCCCTCTTCTCT CGATGACACAGAGCATGATGA	0.416	ttttttttGCATACCGGCCTCTCCC	Forward	16	23	0.1	57.3	G/A
rs12896399 (3F and 3R)	3	14 – 91843416	*SLC24A4*	104	CTGCCGATCCAATTCTTTCT CTTAGCCCTGGGTCTTCATG	0.416	ttttttttttttttttttttttttttttaTCTTTAGG TCAGTATATTTTGGG	Forward	23	53	0.15	54.5	G/T
rs16891982 (4F and 4R)	4	5 – 33987450	*SLC45A2* (*MATP*)	128	TCCAAGTTGTCCTAGACCAGA CGAAAGAGGAGTCGAGGTTG	0.416	ttttttttttAAACACGGAGTTGATGCA	Forward	18	29	0.5	55.9	C/G
rs1393350 (5F and 5R)	5	11 – 88650694	*TYR*	80	TTCCTCAGTCCCTTCTCTGC GGGAAGGTGAATGATAACACG	0.416	ttttttttttttttttttttttttaTTTGTAAAAGA CCACACAGATTT	Reverse	23	47	0.1	55.6	T/C
rs12203592[a] (6F and 6R)	6	6 – 341321	*IRF4*	115	ACAGGGCAGCTGATCTCTTC GCTAAACCTTGGCACCAAAAG	0.416	ttttttttttttttaTTTGGTGGGTAAAAGAAGG	Forward	19	35	0.3	55.2	C/T

Table 1: SNP markers used in the IrisPlex assay for eye colour prediction. * changes made compared to initially published protocol [5]: alternative direction of single base extension SNP primer used

and rs12203592 (*IRF4*) according to our previous description [5]. All marker details and primer sequences, including slight alterations to the previously published protocol, are listed in Table 1. The protocol consists of a single multiplex two step PCR using 1 µl genomic DNA extract (varying concentrations) and primers in a 12 µl reaction which includes 1 x PCR buffer, 2.7 mM $MgCl_2$, 200 µM of each dNTP and uses adjusted thermocycling conditions for increased specificity: (1) 95 ˚C for 10 min, (2) 33 cycles of 95 ˚ C for 30 s and 61 ˚C for 30 s, (3) 5 min at 61 ˚C. This is followed by product purification and a further multiplex single base extension (SBE) reaction using the ABI Prism ® SNaPshot kit (Applied Biosystems) as previously published [5]. All cleaned products were analysed on the ABI 3130*xl* Genetic Analyser (Applied Biosystems) with POP-7 on a 36cm capillary length array. Run parameters were optimised to increase sensitivity, with an injection voltage of 2.5 kV for 10 s, and run time of 500s at 60ºC.

Sensitivity, peak height balance & consistency

The sensitivity of the assay was previously published at 31 pg, for full 6-SNP profiles [5]. Here we repeat this study to assess the impact of the minor changes to the assay. Samples from 3 individuals were prepared by serial dilution from 500 pg and re-measured with the Quantifiler Human DNA Quantification kit in duplicate. Brown, blue and intermediate eye colour phenotypes were genotyped at 500, 250, 125, 62, 31 and 16 pg DNA input with the IrisPlex assay to assess the sensitivity. To ensure consistency of genotyping with the ABI3130*xl*, DNA samples from 3 individuals with differing genotypes and eye colour phenotypes were amplified at the optimum concentration of 250 pg and run 25 times.

Homozygote and heterozygote peak height ranges were noted for each locus. The average peak height at each allele, and heterozygote peak height ratios for each SNP locus were also ascertained at DNA inputs of 1000, 250 and 100 pg. The heterozygote peak height ratio was calculated by dividing the peak height of the lower molecular weight allele (first peak at the SNP locus) by the peak height of the higher molecular weight allele (second peak at the SNP locus) for all SNPs.

Mixture studies

DNA mixtures were made in ratios of 1:1, 1:5 and 1:10, with a total concentration of 500pg. Combinations of DNA from blue: brown, blue: blue and brown: brown eyed individuals were created to assess the impact of multiple sample donors on the IrisPlex systems' ability to predict eye colour.

Species specificity

We examined the specificity of amplification by genotyping a variety of animal DNA samples with the IrisPlex assay. Samples include cat, dog, mouse, rat, bovine, pig, and chicken at DNA amounts of 100 ng and a chimpanzee sample of 1 ng. The DNA was obtained commercially from Novagen, Inc. (Madison, WI) for all but the chimpanzee sample, which was used and described elsewhere [7].

Reproducibility

Concordance testing was performed on 40 DNA samples of varying concentrations and typed by three independent laboratories, two of which had no previous experience with the IrisPlex assay.

Case-type samples & stability studies

Simulated casework samples were genotyped using the IrisPlex assay under a blind proficiency test, as shown in Supplementary Table 1. Samples include blood, semen, saliva, hair, inhibited samples (intentional haem inhibition) and touched items. For the stability study, DNA from one individual was subject to DNase treatment (10 units) at intervals of 5, 10, 20, 30, 40, and 50 minutes and genotyped to test the stability of the assay under degraded conditions, often observed in casework samples.

Eye colour prediction model

The human eye colour prediction model used, has been previously published [2, 5] and is a core component of the IrisPlex system. Statistical prediction probabilities are assigned to each of the SNP alleles obtained from the IrisPlex genotyping assay, based on 3804 Dutch individuals in the model-building set of a previous study [2]. When combined in the model, it gives three probability estimates for brown, blue and intermediate eye colour of the unknown donor, with the highest probability being the predicted phenotype. The model parameters used for this 6-SNP model can be found in Supplementary Table 2. An Excel macro was created to allow simplified calculation of probability estimates, and can be found in Supplementary Table 3.

Population studies

The IrisPlex assay was performed on the HGDP-CEPH H952 samples which includes 952 individuals from 51 worldwide populations and was previously published by this group [5].

RESULTS & DISCUSSION

Multiplex design & protocol

The multiplex design of the IrisPlex assay was altered from its previously published version [5] in a bid to increase its sensitivity and specificity at the lower concentrations of DNA commonly found in casework samples. The annealing temperature of the multiplex PCR was increased slightly for improved specificity, and the SNP primer directions for rs1800407 and rs12203592 were altered in the subsequent SBE reaction to increase peak heights at lower template amounts. In the initial design, the reverse primer at rs12203592 caused a sporadic artefact that affected genotyping with low template levels. The use of the forward primer in the current protocol, giving a C/T genotype, avoids this problem. The change in primer direction of rs1800407 now produces increased peak heights with decreased primer input, which improves the call accuracy of this SNP at DNA amounts less than 250 pg. It is also easier to recognise heterozygote genotypes at this locus due to this increase in peak height. The primer concentration for SNP rs16891982 was also increased from 0.22 µM to 0.5 µM as the homozygote C/C allele was difficult to call with the previous protocol in low concentration DNA samples, due to its considerably lower peak height in comparison to the G/G homozygote allele. Notably, the increase in primer concentration creates a more balanced profile when the C/C allele is present. Finally, the ABI 3130*xl* Genetic Analysers' standard protocol was altered to increase detection sensitivity by increasing injection voltage and time, and to decrease overall processing time with a reduction to a 500 s run time. Overall, the slight changes made

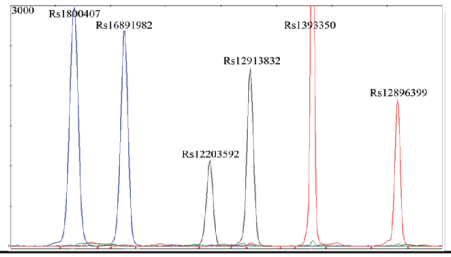
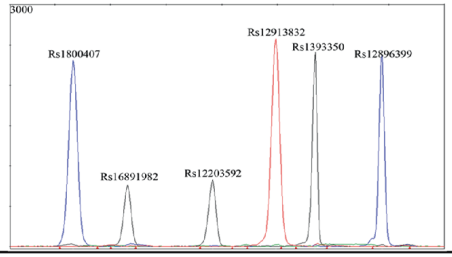
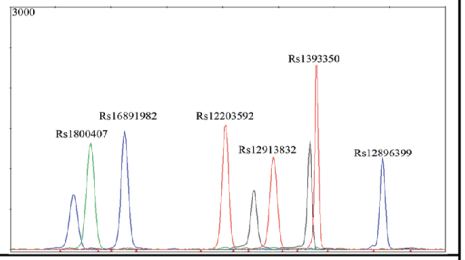
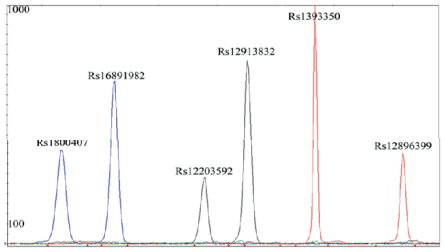
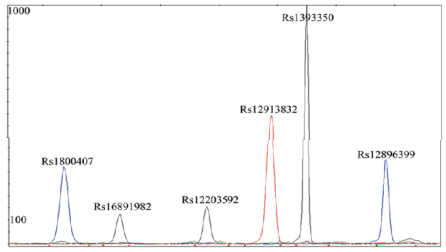
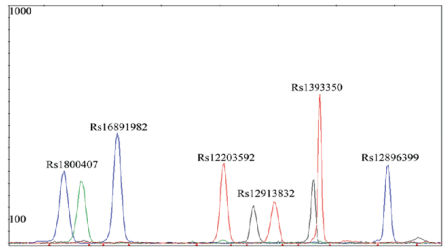
to the previously described protocol [5] enhance the IrisPlex assay performance.

Sensitivity, peak height balance & consistency

IrisPlex genotyping in serial DNA dilutions from individuals with different eye colour phenotypes and genotypes was repeated with the altered protocol, aiming to confirm its high sensitivity as described previously [5]. As evident from Figure 1, we obtained high sensitivity in all samples with complete SNP genotypes down to 31 pg of DNA input, equivalent to approximately six human diploid cells in agreement with results from our initial protocol [5]. Notably, peak heights were increased with the altered protocol providing higher robustness in the genotyping. However, the sensitivity threshold did not change; partial profiles were observed at 16 pg of DNA in several amplifications, with dropout occurring at homozygote SNP rs12203592 in the brown and heterozygote rs1800407 and rs12913832 in the intermediate eye coloured samples, where peak heights fell below the 50RFU threshold for genotype calls (the commonly used standard in STR typing). Allele peaks were only called if they were above 50RFU in their respective size range within a custom designed IrisPlex bin set, using the GeneMapper 3.7 software (Applied Biosystems). Increased allelic dropout from 16 pg was particularly noted at heterozygous SNPs, as could be expected due to their lower effective template amount compared to homozygous loci. Overall, the achieved sensitivity with these new changes is comparable to the initial protocol published [5] which is relatively higher in comparison to several autosomal SNP assays currently being used, such as the 49 SNPs analysed

in two SBE multiplexes based on the SNP*for*ID 52plex by Sanchez *et al*. [8] which requires a minimum of 80 pg for a full profile [9]. It also surpasses the commercially available AmpF*l*STR Minifiler kit (Applied Biosystems) in terms of sensitivity, which requires at least 125 pg of input DNA for a full profile [10]. As there are a lower number of SNPs used in this assay, and an increased number of PCR cycles, this does contribute to the higher level of sensitivity observed. However overall, as a forensic application, the particularly high level of sensitivity of the IrisPlex system will be of great benefit to many difficult investigations, where the quality and quantity of the casework samples is quite low.

One important aspect of sensitivity testing with SNPs is heterozygote alleles, which we illustrate in Figure 2 and 3. Figure 2 shows the peak height range observed in homozygotes at 250 pg, which falls into the assay's optimum range (250-500 pg), and revealed little deviation from their respective averages at this level of DNA input, with the exception of rs1800407G.

DNA input (pg)	Blue Individual (500 pg)	Brown Individual (500 pg)	Intermediate Individual (500 pg)
			
	Blue Individual (31 pg)	Brown Individual (31 pg)	Intermediate Individual (31 pg)
			
500	√	√	√
250	√	√	√
125	√	√	√
60	√	√	√
30	√	√	√
15	√	X	X

√ = full profile observed X = drop out of one or more allele

Figure 1: Sensitivity testing of the IrisPlex assay for eye colour prediction of phenotypic blue, brown and intermediate eye coloured individuals ranging from 500 to 16 pg DNA input, with visual profiles at 500 pg and 31 pg DNA input.

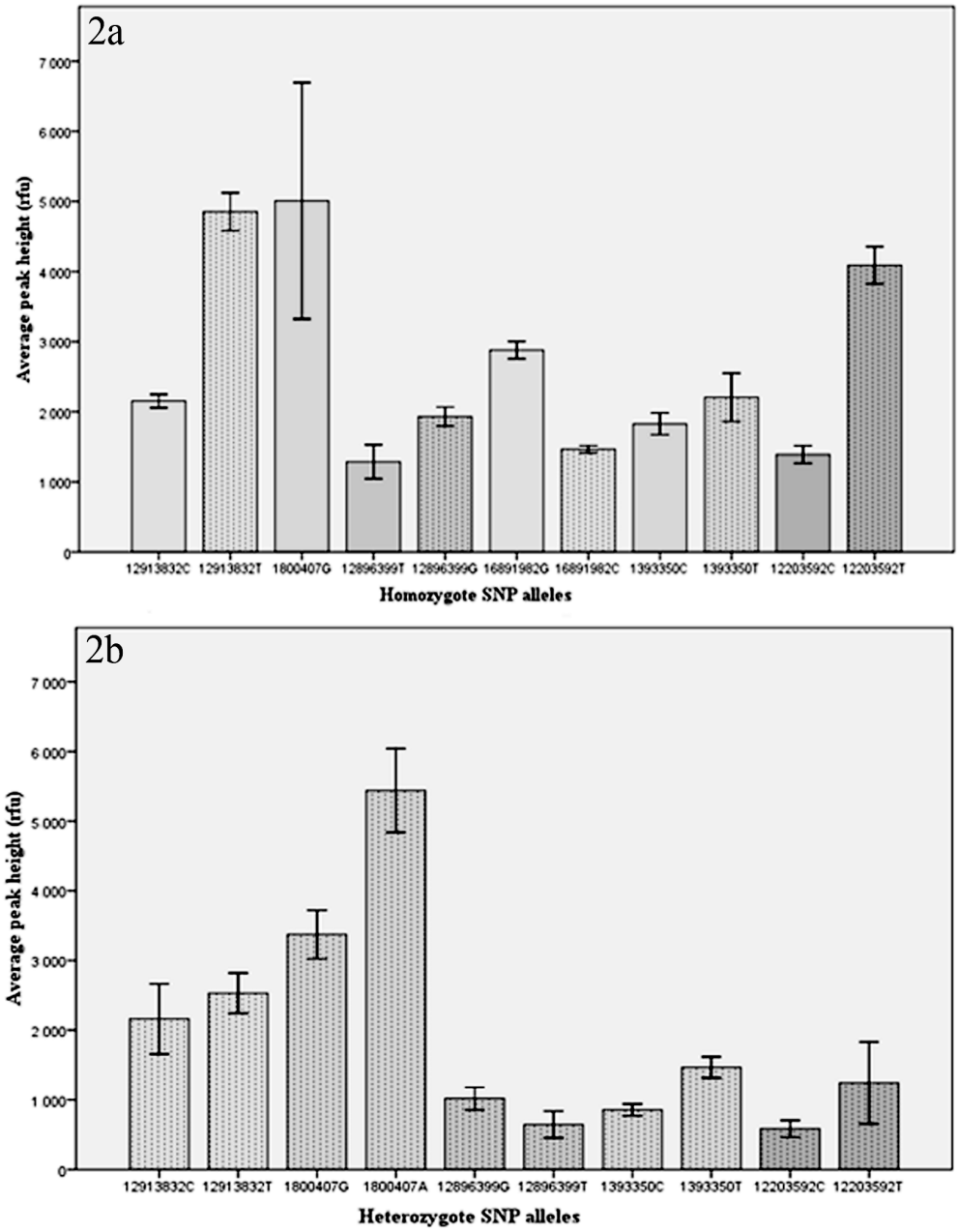


Figure 2: Homozygote and heterozygote average peak heights from 25 replicates of 250 pg of DNA input for each allele in the IrisPlex assay, run with an ABI 3130*xl* Genetic Analyser, using POP-7 on a 36cm capillary length array. The rs1800407A allele and the rs16891982 heterozygote peaks are absent from 2a and 2b parts of the figure respectively. Error bars represent 95% standard error of the averages.

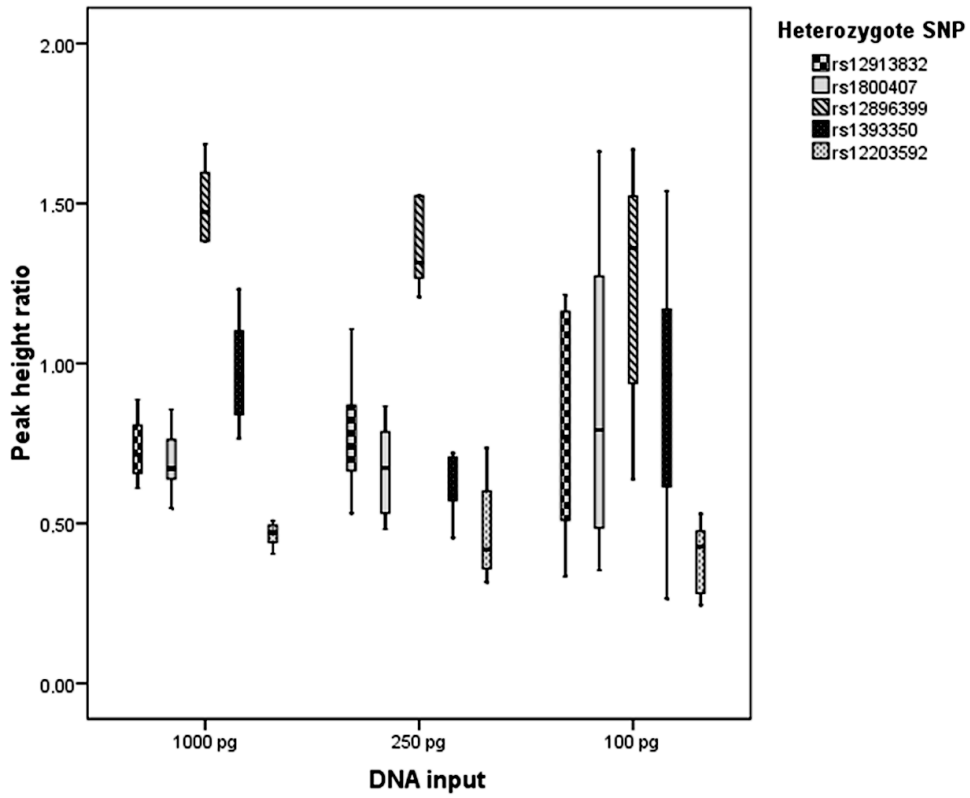


Figure 3: Box-whisker plot with heterozygote peak height ratios of the IrisPlex SNPs at DNA input levels of 1000 pg, 250 pg and 100 pg, showing the increase in variability with decreasing template amount. Rs16891982 is not present due to lack of a heterozygote sample.

This is due to the highly sensitive nature of this SNP in the assay; despite the primer concentration being low, it consistently yields higher peaks than the other SNPs. However, the large range observed does not reduce the genotyping success of the assay, as the minimum peak height observed in 25 replicates was 3000 RFU, and as such, in its homozygote state, the locus is not subject to dropout. Unfortunately, average peak heights could not be recorded for the A/A homozygote allele for this SNP, due to its extreme rarity. In figure 4(b) of our previous paper [5],

rs1800407 worldwide allele distributions demonstrates that the A/A homozygote allele was found only in Europe and Central South Asia at frequencies of 0.7% and 1% respectively, and therefore we did not have enough examples of this SNP in the present validation study. Figure 2b of the present article displays the range of heterozygote SNP peak heights at 250 pg. The heterozygote SNP at rs12913832 displays the most even amplification between alleles, but the other SNPs show preferential amplification of one allele in heterozygotes. This is a common feature of fluorescently labelled SNP analysis, due to the inherent differences in intensity levels between the four dyes used to label the four extended bases. The heterozygote SNP profile of rs16891982 is not represented in this figure due to its rarity and thus sufficient data could not be collected. Figure 4d from our previous paper [5] illustrates the worldwide allele distribution of rs16891982, which displays the homozygote G/G allele limited to Europe and the homozygote C/C allele common in the rest of the world, with little presence of the heterozygote genotype.

Peak height ratios between heterozygous alleles are examined further in Figure 3, which displays the variation in peak balance across several DNA template amounts. Equal peak heights at homologous alleles give a balance ratio of 1, while 0.5 represents the smaller molecular weight (first) allele having a peak height half that of the larger molecular weight (second) allele, and the converse for a ratio of 2. From 1ng of template DNA, the ratio of peak heights observed between the alleles is comparatively consistent – although rs12896399 and rs12203592 have unbalanced amplification, the direction and strength of imbalance is constant across all amplifications. However, more variability was seen in both peak heights and peak balance with lower template amounts, as

could be expected due to inherent stochastic variation. Amplification bias is no longer constant at 100 pg of DNA template, with ranges in peak height ratio between 0.31 and 1.67. Overall, although one should be cautious when using very low levels of template DNA, it is possible to assess the quality of an IrisPlex amplification based on peak height ratio, which has also been observed in other studies involving the quality of amplification using peak height ratios [11].

Mixture studies

Mixture studies were performed to assess the probability of detecting a 2-person mixture with the IrisPlex assay. It has been noted that mixture recognition can be difficult with biallelic SNPs [12], particularly with low numbers of markers, therefore the presence of a mixture is more finely determined by STR analysis. As standard crime scene sampling ensures STR typing prior to any downstream processes such as the use of the IrisPlex, it is the preferred method of mixture determination, however we illustrate here, for added informative purposes, some interesting factors that may suggest the presence of a mixture using the IrisPlex assay. As the IrisPlex SNP alleles are specifically associated with particular eye colours, it can be difficult to detect a mixture composed of 2 individuals with the same phenotype (blue:blue or brown:brown mixtures, data not shown), although some genotype variation within categorical eye colour phenotypes exists. Nonetheless, in most cases the combination of alleles and resultant statistical prediction probability would return the correct eye colour prediction, due to the multiple donors sharing the same phenotype. However, a 1:1 blue:brown mixture (Figure 4) may be

detectable, based on the low frequency of the heterozygous genotype at rs16891982, as discussed above. Although this cannot be seen as definitive proof of a mixture, it may indicate that further testing of a mixture in the DNA sample is necessary, if it has not already been performed. The low frequency of the heterozygote may be explained by its location in the SLC45A2 gene, which is strongly associated with pigmentation and has been linked to population origin [13]. The strong geographical separation between the two homozygote genotype distributions suggest that any heterozygote genotype result should be examined further using STRs or other markers to exclude the probability of a mixture. Apart from the rs16891982 SNP, it is not possible to detect a mixture using the IrisPlex assay at a 1:1 mixture ratio due to the low number of SNPs in the assay. However, there are other indications of sample mixture seen in 1:5 and 1:10 ratio examples. As seen in Figure 4, rs12913832, rs1393350 and rs12896399 display substantially lower peak heights for the usually predominant allele in single source heterozygote genotypes, and therefore it is easy to recognise that there may be complications with the profile and that a mixture may be present. Overall, although it is not possible to differentiate SNP allele profiles with this assay, the well-characterised average peak heights and peak height ratios presented here allow the possible confirmation of the presence of a mixture in an unknown sample, as standard STR typing would have shown a mixture initially, the IrisPlex assay may verify this to prevent an inaccurate eye colour prediction probability from being made. As standard protocols involve STR typing of crime scene samples, that is the preferred method of mixture determination rather than the IrisPlex assay

alone, especially at 1:1 mixture ratios, where individual autosomal SNP profiles would be indiscernible.

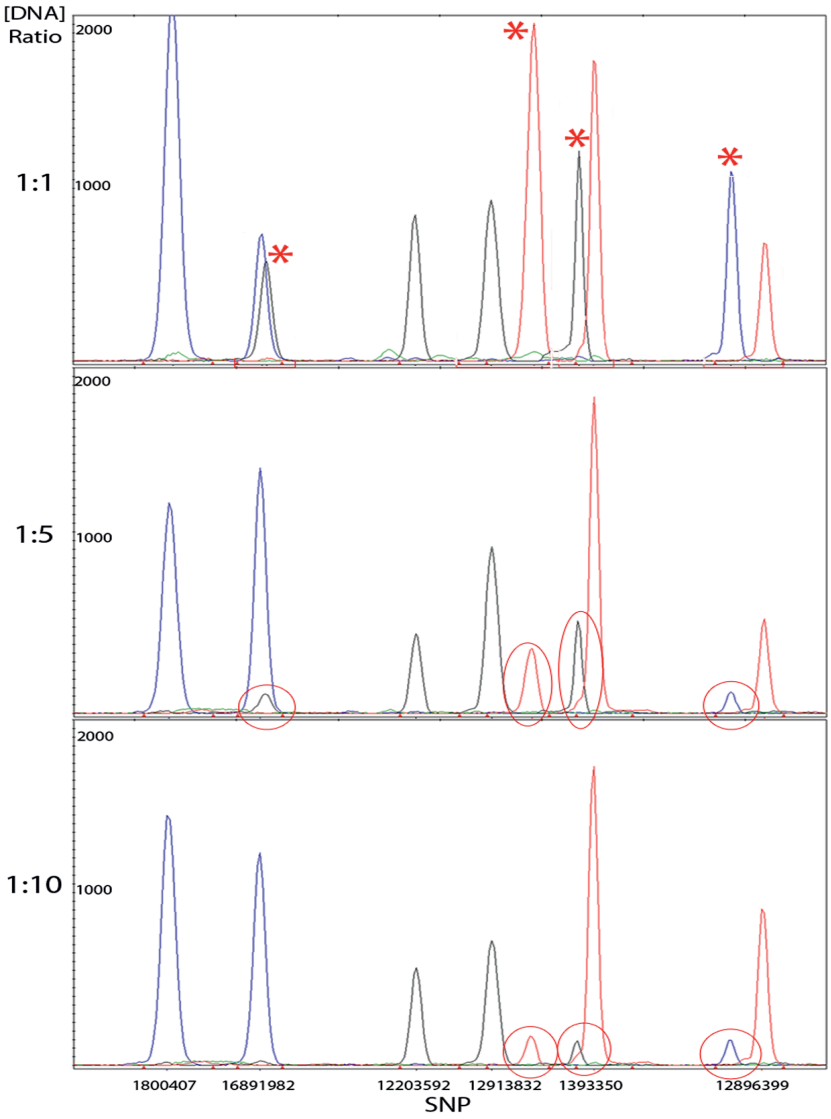


Figure 4: Mixture study of 1:1, 1:5 and 1:10 from DNA samples of two individuals, with brown and blue eye colour phenotypes, respectively. The total volume of DNA used is 500 pg. The asterisks represent the brown eye colour phenotype contributing alleles, which are also highlighted with a circle at ratios 1:5 and 1:10 indicating the abnormal peak height ratio between alleles, at which a mixture may be detected.

Species specificity

The IrisPlex assay was tested with samples from several animal species to assess its human specificity. From all species tested, only the chimpanzee gave a full SNP profile, with a strongly predicted brown eye colour as expected. The most predictive SNP, rs12913832, is strongly conserved across primate, ape and lemur species in the brown allelic form [14]. However, it appears that the human blue genotype differs from other non-human primates, which also show the blue eye colour phenotype, at least at this SNP [14]. Other species commonly encountered in casework, such as cat, dog, mouse, rat, bovine, pig, and chicken, did not show amplification of the full set of IrisPlex SNPs. Supplementary Figure 1 displays the peaks observed and called above the 50 RFU threshold in each of the species at 100 ng. Notably, the majority of the species in this list did not display any allele peaks, and the rest, only produced one peak with low intensity values even at 100 ng DNA input. As such, the species specificity of the IrisPlex assay is sufficient for routine casework situations. However, more investigation of the degree of SNP conservation across higher primates and monkeys may be of value.

Reproducibility

Concordance testing of the IrisPlex assay was performed by 'blind testing' a panel of 40 samples of varying concentrations and types across three independent laboratories. The two external laboratories involved were the Forensic Laboratory for DNA Research (FLDO) at Leiden University Medical Centre and the Netherlands Forensic Institute (NFI), both involved in routine forensic DNA analysis. One test site (FLDO) had previous experience with SNaPshot chemistry, while the other (NFI) had not

performed SNP genotyping or SNaPSHOT prior to the IrisPlex validation testing. Both were provided with DNA samples, a written protocol as described above in the Materials and Methods, and the Excel macro for statistical prediction of eye colour (Supplementary Table 3). Thus, the blind test investigates the reliability and simplicity of the assay when performed by laboratories and individuals with no experience of the IrisPlex system. Supplementary Table 4 provides full descriptions of the samples tested, and the level of concordance between the three laboratories. The majority of the samples were genotyped correctly in both external laboratories, displaying the ease and consistency of newly implementing the IrisPlex assay for eye colour SNP genotyping and phenotype prediction. The only samples that had discordant results were the mixture samples (where mixed samples were called as single source) and one laboratory had difficulty with the lowest level DNA input samples, possibly due to evaporation or freeze/thaw of these samples. As discussed previously, it is possible to detect mixtures (e.g. more than 1:5 ratios) with the IrisPlex system, although some experience with average peak heights and ratios is required, which was not yet available in the external test sites for this specific assay. However, even without this experience, and without knowledge of the rarity of certain SNP genotypes, both laboratories commented that further testing of a number of the mixed samples was required due to the unusual peak height ratio, before a phenotype probability could be predicted. However, as phenotype prediction is downstream in the analyses process of a crime scene sample, the presence of a mixture would more than likely be known prior to the use of the IrisPlex system. In general, even with the different experience levels in

using this technology and SNP assays, both laboratories commented on the consistency and ease of use of the IrisPlex system.

Case-type samples & stability studies

Simulated casework samples were created to test the IrisPlex assays range and ability to produce a correct phenotype prediction from varying sample types with differing qualities and quantities, as is often found in crime scene samples. The samples were also blind-tested to check the proficiency level of the validation. Supplementary Table 1 gives a full list of the types of samples tested, their concentration and the profilers' blind test results. All mixtures (touched items) and single source samples (blood, saliva, semen, hair, touched), when extracted with sufficient DNA quantities, were genotyped correctly and the eye colour phenotype prediction was wholly accurate for single source samples. Thus, the IrisPlex system is able to accept a range of sample types, and showed 100% accuracy across a number of casework type samples. Single source DNA was also treated with 10 units of DNase I (Roche) for varying time intervals to simulate DNA degradation to measure how the IrisPlex assay copes with such samples. The assay was specifically designed to facilitate the amplification of template less than 130bp in size; in this way amplicon sizes are less than the observed 146bp protection limit of the 'core particle' nucleosome complex [15], giving increased probability that amplification will be successful even under extreme degradation conditions. Full profiles were observed at 5, 10, and 20 minutes DNase activity, with a complete lack of amplification after 30 minutes. This was most likely due to the lack of DNA template amplification during the first PCR reaction of the SNP flanking regions, and the near complete

degradation of the DNA molecule (less than 87bp). Due to its design, the ability to obtain full profiles from DNA with up to 20 minutes of strong DNase activity shows how robust the IrisPlex assay is at genotyping degraded DNA and is comparable to other validated autosomal SNP assays [8].

Eye colour prediction model

One of the main components of the IrisPlex system, the eye colour prediction model, was previously developed by Liu *et al*. [2] and is based on a multinomial logistic regression model. The model parameters were derived in the previous study, which contained 3804 Dutch European individuals and the result, based on an individual's genotype, yields probabilities of being brown, blue and intermediate eye colour, with the highest probability being the most informative [2]. In our previous study [5] a 6 SNP statistical model was applied using genotype results from 40 individuals from several continental regions with known eye colour phenotypes (the model parameters can be found in Supplementary Table 2). We found that using a threshold of ≥0.7 as a true blue or brown eye colour probability gave an eye colour prediction accuracy of 100%. Other threshold levels could also be used (i.e. ≥0.5p), although with this value 3 out of 40 phenotype calls were incorrect, so therefore the conservative value of 0.7 for true blue or true brown eye colour as a cut-off is preferred. Values below this threshold were called 'undetermined', although this may include eye colours such as hazel brown and blue/grey, which are not defined as true brown or true blue. As yet, SNPs specifically associated with these intermediate eye colours have not been identified, which

lowers the ability to predict these colours with complete accuracy. In our experience, individuals with the intermediate brown and blue eye colours will have the correct prediction made, although the probability will be lower (generally between 0.5 and 0.7). Ideally, large-scale genotype/phenotype analysis must be performed to gather more information on sensitivity and specificity values of lower thresholds, but at present with all available data the probability threshold of 0.7 is determined to be the most efficient. A user-friendly interactive spreadsheet in which the genotypes can be entered to yield the eye colour prediction probability can be found in Supplementary Table 3.

Population studies

The IrisPlex system was tested on the HGDP-CEPH H952 worldwide samples as a population study and was previously published by this group [5]. In summary, the IrisPlex assay gave the genotypes of 934 individuals from 51 worldwide populations (several samples could not be typed due to low DNA quantity), and using the statistical model with the recommended threshold level of ≥0.7p for eye colour prediction, an eye colour map of the world can be seen [5]. It shows the restriction of predicted blue eye colours (≥0.7p) to Europe and immediate surrounding areas (Middle East and West Asia) whereas in the rest of the world, only brown eye colour (≥0.7p) was predicted. This predicted worldwide eye colour distribution fits with the expected trend, given existing general knowledge and available data of eye colour variation around the world [16], although eye colour phenotypes were not available for the genotyped samples. This displays the accuracy and reliability of the

IrisPlex system in the prediction of true blue and true brown eye colour individuals outside of Europe, independent of knowledge on the bio-geographic ancestry of the sample donor, which may not be available, although more worldwide data with phenotypes should be typed to confirm this.

CONCLUSIONS

The developmental validation of the IrisPlex assay, as part of the IrisPlex system, fulfils all SWGDAM guidelines in terms of species specificity, sensitivity, stability, and mixture studies, reproducibility, precision and accuracy, casework samples and population studies. Due to its design, it surpasses many genotyping assays currently used in the forensic context in terms of sensitivity, with 31 pg minimum DNA input, partly due to the increased PCR cycling protocol in comparison to commercial STR kits. It is capable of producing accurate results from simulated casework samples that include blood, semen, saliva, hair, and trace DNA samples, including extremely low quantity samples. It also produces consistent results in both internal and external laboratory investigations with extreme ease of use and reliability. In conjunction with the human eye colour prediction model, based on data from thousands of Europeans and provided in an easy to use excel macro tool for prediction probability estimation, the IrisPlex system is ready for immediate implementation and use in any accredited forensic laboratory for aiding DNA intelligence investigations. For example, due to the expected revision of Dutch legislation, this will allow the use of the IrisPlex eye colour prediction system in Dutch forensic casework as of 2011.

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Supplementary Material can be found at

http://www.sciencedirect.com/science/article/pii/S1872497310001614

Or the **APPENDICES** of this Thesis.

CHAPTER 2.3

DNA-based eye colour prediction across Europe with the IrisPlex system.

Forensic Science International: Genetics 6, 330-340 (2012)

ABSTRACT

The ability to predict Externally Visible Characteristics (EVCs) from DNA, also referred to as Forensic DNA Phenotyping (FDP), is an exciting new chapter in forensic genetics holding great promise for tracing unknown individuals who are unidentifiable via standard forensic short tandem repeat (STR) profiling. For the purpose of DNA-based eye colour prediction, we previously developed the IrisPlex system consisting of a multiplex genotyping assay and a prediction model based on genotypes and phenotype data from 3804 Dutch Europeans. Recently, we performed a forensic developmental validation study of the highly sensitive IrisPlex assay, which currently represents the only validated tool available for DNA-based prediction of eye colour in forensic applications. In the present study, we validate the IrisPlex prediction model by extending our initially described model towards genotype and phenotype data from multiple European populations. We performed IrisPlex analysis on 3840 individuals collected at seven sites across Europe as part of the European Eye (EUREYE) study for which high-resolution eye images were available. The accuracy rate of correctly predicting an individual's eye colour as being blue or brown, above the empirically established probability threshold of 0.7, was on average 94% across all seven European populations, ranging from 91% to 98%, despite the large variation in eye colour frequencies between the populations. The overall prediction accuracies expressed by the area under the receiver characteristic operating curves (AUC) were 0.96 for blue and 0.96 for brown eyes, which is considerably higher than those established before. The IrisPlex prediction model parameters generated from this multi-population European dataset, and thus its prediction capabilities, were highly comparable to those previously

established. Therefore, the increased information regarding eye colour phenotype and genotype distributions across Europe, and the system's ability to provide eye colour predictions across Europe accurately, both highlight additional evidence for the utility of the IrisPlex system in forensic casework.

INTRODUCTION

People of European descent display the widest variation in pigmentation traits, such as iris (eye) and hair colouration, in the world. In particular, eye colour variation is nearly restricted to people of (at least partial) European descent. Eye colour categories here often concern blue, brown and intermediate (green etc.). In the rest of the world, people tend to have brown eye colour, which is considered to be the ancestral human trait in agreement with the Out-of-Africa hypothesis of modern humans. The current variation in eye colour is thought to have originated via a genetic founder event involving non-brown irises in early European history [1, 2]. It is furthermore assumed that eye colour variation in Europe has been shaped by positive selection via sexual selection i.e., mate choice preference [1, 3]. Alternatively it has been proposed that eye colour variation evolved via a correlation with skin colour and its environmental adaptation e.g. maximizing vitamin D conversion in low levels of UV radiation [1, 3], or as a combination of both. One suggested geographic region for the origin of blue eye color in Europe is the southern Baltic, as indicated by concentric rings of decreasing frequency of the blue eye colour trait spreading from the southern Baltic region, resulting in a strong north-south gradient in blue eye colour frequency across Europe [1, 3, 4].

Several recent genome-wide association (GWA) and candidate gene studies using European populations found the *HERC2* and the *OCA2* genes to be the most involved in human eye colour variation [2, 5-9], principally concerning the two extremes of brown and blue iris colour. In particular, the single nucleotide polymorphism (SNP) rs12913832 in *HERC2* explains most blue and brown eye colour variation [2, 6-7, 9-12]. It has been hypothesized that this intronic SNP is thought to provide a

regulatory effect to the neighbouring *OCA2* gene [5, 6-8], but reliable functional data evidence is unavailable thus far. In addition, there are several other genes known to have more minor effects on human eye colour variation, such as *SLC24A4* [8, 13], *TYR* [5], *TYRP1* [13], *SLC45A2* [8], *IRF4* [8], *ASIP* [14], and more recently shown *LYST* [15] and *DSCR9* [15]. For a recent review on the genetics of human iris colour, see Sturm et al. [11].

Although the exact functional effect of many eye colour associated SNPs is currently unknown, we previously found in a systematic study considering 37 eye-colour associated SNPs from 8 genes in 6168 Dutch Europeans from the Rotterdam Study, that blue and brown eye colour was accurately predicted by 15 SNPs from 8 genes [9]. Of those, 6 SNPs from 6 genes cover almost all the predictive eye colour power provided by using all the SNPs tested [9]. Based on this knowledge, we recently developed the IrisPlex system for accurate prediction of blue and brown eye colour from DNA [16]. One component of the IrisPlex system consists of a highly sensitive 6-SNP multiplex SNaPshot assay, which we recently successfully validated for forensic applications, demonstrating that it complies with strict SWGDAM (Scientific Working Group on DNA Analysis Methods) guidelines [17]. The other component of the IrisPlex system is a prediction model based on genotype and phenotype data from 3804 Dutch Europeans, allowing straightforward and user-friendly eye colour probability estimation based on an excel spreadsheet made available with the previously published article [16]. Furthermore, we previously demonstrated in a worldwide IrisPlex analysis that the ability of the IrisPlex system to predict a person's categorical eye colour reliably is independent of bio-geographic ancestry; hence, no prior knowledge

about the geographic region of genetic origin is required for IrisPlex application [16].

MATERIALS & METHODS

Subjects, images, genotyping, and eye colour classification

Previously extracted DNA samples were obtained from the EUREYE study. EUREYE is a population-based study of age related macular degeneration in seven centres located across Europe. Participants were recruited from random sampling of the population aged over 65 years in the centres: Bergen (Norway), Tallinn (Estonia), Belfast (UK), Paris-Creteil (France), Verona (Italy), Thessaloniki (Greece), and Alicante (Spain). The great majority of participants were of European origin who were born in the country of the participating centre. The only exception to this was Paris-Creteil where 17% of the centres participants were born in North Africa, however as we did not have data on their ethnic origin, we were unable to ascertain if they were of European or Arabic descent. Participants were interviewed by fieldworkers, underwent an eye examination including digital capture of the iris and provided a blood sample for DNA analysis. Study participants gave informed written consent; the study was approved by national ethical committees and met the criteria of the Helsinki declaration. A detailed description of the EUREYE study including image collection can be found elsewhere [18, 19]. In brief, iris photography involved illumination of the anterior segment of each eye to show the colour of the iris with a flash intensity of 25 to 36mW using a Topcon TRC 50 EX camera under normalised conditions. The photographs were checked for bleaching and inclusion of the pupillary margins, images were repeated if necessary. Multiple images were taken of both left and

right eyes for each participant, with and without pupillary dilation. Each digital eye image was classified (sample ID was masked) as blue, brown or undefined by a single grader (SW) and the individuals final eye colour category was then classified based on the results of all of the individual's images together. This ensured accuracy of eye colour category determination because if there were discrepancies between the photographs, a further examination of all the individuals' eye images took place until a final category was chosen. The grader tried to limit as much variation as possible by assessing the images in a controlled environment using the same computer screen with the same lighting. To be classified

	Perception of green elements	Distinct pupillary rings of alternative colours	Other colours including golden yellow/ orange amber	Total numbers graded 'undefined'	Numbers graded overall
Norway	8	18	11	37	547
Estonia	8	15	10	33	579
UK	7	17	14	38	498
France	3	30	14	47	616
Italy	10	33	20	63	542
Greece	0	8	8	16	547
Spain	1	13	19	33	511
Overall	*37*	*134*	*96*	*267*	*3840*

Table 1: Description and frequencies, per sampled European population, of the types of eye images that were assigned to the 'Undefined' category within the EUREYE data set

within the blue or brown categories, the majority of irides from the multiple images of each eye must have been clearly composed of only one colour, although the intensity of the colour did not affect classification. In contrast, the third category called the 'undefined' category in phenotypic classification, consisted of both green-eyed individuals, and those with two or more pigments within the iris that could not be classified into just one blue or brown category. Examples include individuals with a large peripupillary ring (i.e. blue iris with a brown ring around the pupil,) that could not be defined as having solely blue or brown eye colour at a distance (Table 1). Heterochromatic individuals that differed in category colour between left and right eyes (i.e. one blue eye and one brown eye) were excluded from the study. Supplementary Table 1 displays the frequencies of phenotypic colour per country within sampled European countries. Both DNA and image-derived eye colour phenotypes were available for 3840 individuals in the EUREYE study (in all other cases, iris images were not available, or of insufficient quality for eye colour classification, or DNA was of insufficient quality). The DNA samples were genotyped using the IrisPlex assay targeting 6 SNPs: rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs12896399 (*SLC24A4*), rs16891982 (*SLC45A2* (*MATP*)), rs1393350 (*TYR*) and rs12203592 (*IRF4*), as previously described [9, 16, 17].

IrisPlex model - probability threshold assessment

To assess the effect of using a threshold on accuracy and call-rates, data were grouped according to the highest predicted eye-colour probability (p) with no threshold and then varying thresholds ranging from ≥0.5 to ≥0.9 were applied in a stepwise fashion of 0.05 increments. In these

groups, summary statistics using the IrisPlex model on the EUREYE data include number of correct calls, incorrect calls, total number of samples with probabilities exceeding the defined threshold, percentage of prediction error rate of total samples over threshold, and percentage loss of samples due to implementation of the threshold (i.e., samples put into the undefined category due to p value lower than threshold). The blue and brown eye prediction categories were also examined separately to determine if individualised category thresholds would be more appropriate. Data mapping was performed using MapViewer 7 (Golden Software Inc., Golden, CO, USA) and statistical analysis, unless otherwise stated, was performed using SPSS PASW version 17.0.2 software (SPSS Inc., Chicago, USA)

Eye colour prediction modelling

To develop a European eye colour prediction model considering data from across Europe, the EUREYE samples were randomly assigned to either an 80% model building set (n=3071) or a 20% model verification set (n=769). We repeated the multinomial logistic regression (MLR) modelling using the method previously published by Liu et al. [9] with the full EUREYE dataset to examine any potential alteration in parameter estimates caused by the inclusion of increased numbers of populations. In brief, with the six SNP genotypes and phenotypes categorised into blue, brown or undefined per individual as input data, the regression model was able to generate α and β parameters based on the predication capability of each minor SNP allele for each colour category for that set. Following the model generation, the parameters were tested with the 20% model verification set. For each individual in the verification set, the

presence of the minor allele at each SNP was entered as 0, 1 or 2 into the model containing the new α and β parameters. Based on the formula provided by Liu et al [9], three prediction probability values were generated for each of the three defined phenotype categories, blue, brown and intermediate iris colour, with the sum of all prediction probabilities equalling 1. The highest probability of all three categories was taken as the predicted iris colour of that individual. An example of the IrisPlex probability calculation tool can be found in our previous publication [16]. Comparison of the two models, namely the original IrisPlex model [9, 16, 17] and the IrisPlex EUREYE model, their associated parameters and prediction accuracy was performed using a ROC (Receiver Operating Characteristic) curve and tested through the use of AUC (Area Under the receiver operating characteristic (ROC) Curves) values for each category and model. The AUC measurement is the integral of the ROC curve and ranges from 0.5 (random prediction) to 1 (perfect prediction) [20], and is prevalence adjusted, so the closer the AUC value is to 1, the more accurate the model is. MLR model building and testing was performed with the freely available statistical software 'R', version 2.11.1 [21] using the packages mlogit [22] for model building and ROCR [23] to produce the ROC curve data. Correlation coefficients were estimated by means of a Pearson correlation co-efficient using SPSS PASW version 17.0.2 for Windows (SPSS Inc., Chicago, USA).

RESULTS & DISCUSSION

Eye colour phenotype distribution across Europe

The EUREYE sampling population represents a wide range of bio-geographic ancestries from throughout Europe. The sampling sites in seven countries (UK, Norway, Estonia, France, Italy, Spain and Greece) are distributed across Europe including northern, western, southern and central parts. Although EUREYE does not allow a dense sampling scheme across Europe, it may serve as a reasonable representation of European eye colour variation. This is indicated by the close correspondence of the established map of iris colour variation from the EUREYE samples used here (Figure 1) with that presented in 1965 in the classical image of eye pigmentation in Europe, produced from early 20th century anthropological studies [4] which has been modernly depicted as available on the Eupedia website http://www.eupedia.com/europe/maps_of_europe.shtml#eye_colour. In particular, there is a gradual increase in frequency of blue-eyed individuals from southern to northern Europe, with three distinct iris colour zones appearing from the EUREYE data. These are: Northern Europe, with Northern Ireland, Norway, and Estonia representing the highest frequencies of blue-eye colour, with an average frequency of 75% (range 71.5% to 78.8%); central Europe consisting of France (28.7%) and Northern Italy (29%) with medium to low blue-eye colour frequencies; and finally southern Europe's Spain and Greece at the lowest average frequency of blue eye colour with 16% and 12.4% respectively. Conversely, brown eye colour decreases from southern to northern Europe as evident from our data (Figure 1) and described before [4]. In the EUREYE data, brown eye

colour is highest in southern Europe with an average frequency of 81% (range 77.5% to 85%) and lowest in northern Europe with on average 17.8% (range 15.5% to 20.9%), whereas in central Europe the frequency is intermediate with an average of 61.3% (range 59% to 63.6%). However, it is naïve to assume that the seven sampling sites are fully representative for their countries / regions and we therefore position the pie charts (Figure 1) at the places (cities) where EUREYE sampling was performed.

The proportion of undefined eye colours, as classified from image inspection also differs across the EUREYE sampling sites, albeit less strongly than the blue and brown colours (Table 1). This may be caused by several factors as different phenotypes with similar distributions are combined in this third category including non-classical eye colours, such as green, together with those heterochromatic individuals in whom both irises look the same but are composed of different colours (i.e. blue with brown ring of colour around pupil) (see methods). For instance, in the southern European sampling sites, especially in Greece where brown eye colour is most frequent, there are fewer individuals categorized with undefined eye colour. This can be explained by the fact that brown-eyed individuals tend to display fewer non-classical phenotypes than blue-eyed individuals (e.g. blue-green). As the blue eye colour frequency increases in mid to northern Europe, there are also increased numbers of these intermediate colours including irides containing both colours, such as a predominantly blue iris displaying peripupillary rings of brown. This phenomenon is known as central heterochromia and is caused by differing amounts of melanin within the melanocytes distributed across the iris [24]. The size of the ring can vary greatly between individuals, and

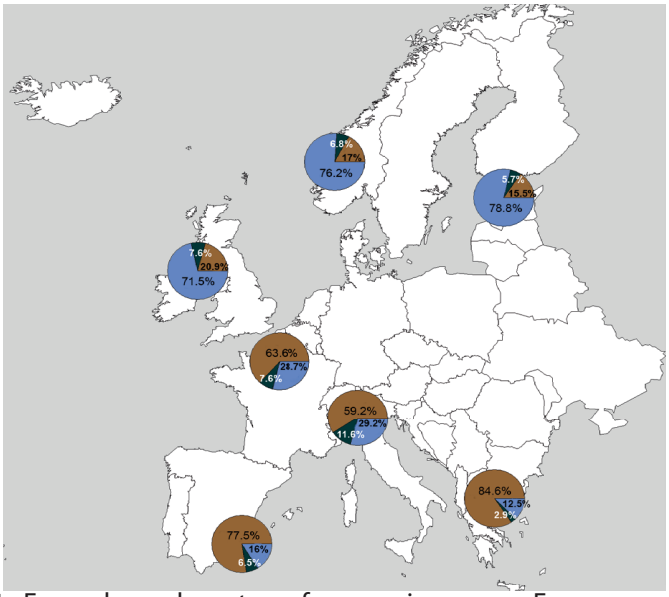

Figure 1: Eye colour phenotype frequencies across Europe representing Bergen (Norway) (N= 547), Tallinn (Estonia) (N= 579), Belfast (UK) (N= 498), Paris (France) (N= 616), Verona (Italy) (N= 542), Thessaloniki (Greece) (N= 547) and Alicante (Spain) (N= 511). Numbers indicate the total number of individuals observed. Within each pie chart, the blue colour represents the frequency of blue eyed individuals, brown represents the frequency of brown eyed individuals, and the black colour represents the frequency of individuals whose eye colour could not be defined clearly into either the blue or the brown category from the image analysis.

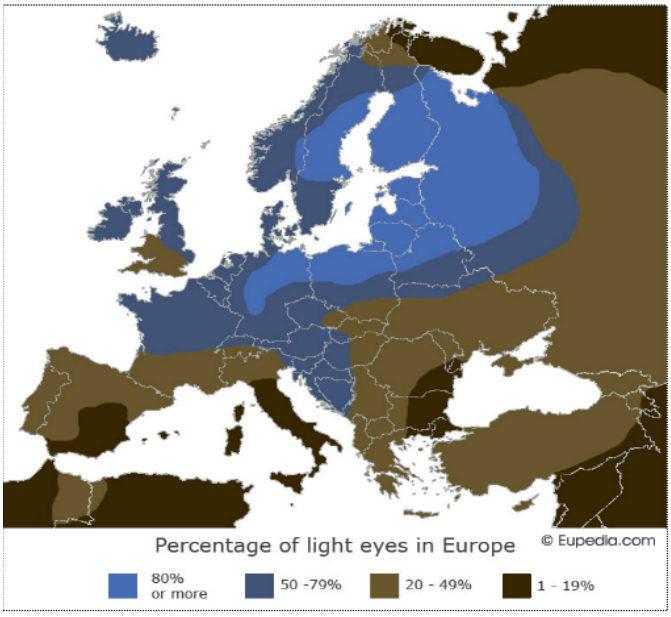

Image taken from the Eupedia website:
http://www.eupedia.com/europe/maps_of_europe.shtml#eye_colour

in some cases be of sufficient size to alter the perceived eye colour from, for example, blue to hazel (light brown).

As such, individuals with prominent peripupillary rings of a colour that opposed the main iris colour were classified into the undefined eye colour category. A breakdown per country of the varying eye colour types assigned to the undefined category can be found in Table 1. The genetic understanding of general and central heterochromia and other iris coloration patterns such as nevi (darkly pigmented spots scattered across the iris), contraction furrows (concentric rings of lighter pigment around the pupil) and Wolfflin nodules (white spots at the edge of the iris, caused by bundles of collagen fibres), as well as of non-classical eye colours such as green, remain to be investigated in future studies.

IrisPlex SNP genotype distributions across Europe

An examination into the worldwide allelic distribution of the six IrisPlex SNPs was previously published [16], and the EUREYE samples now provide an opportunity to improve the data resolution within Europe (Figure 2). The currently available most eye colour predicting SNP, rs12913832 from the *HERC2* gene, which was previously identified as rank 1 in an eye colour prediction analysis involving 24 SNPs [9], shows a strong geographic pattern in genotype distribution across Europe as may be expected (Figure 2, Panel A). There is a clear decline in the frequency of the ancestral brown-eye associated TT genotype from southern to northern Europe, and a corresponding increase in the frequency of the derived blue eye colour associated CC genotype towards northern Europe. A striking correlation also exists between the latitude map co-ordinates of the sampling sites and the increase in frequency of the derived C allele for this

SNP in the south to north direction (r^2 = 0.95, P<0.01). This lends additional support to the hypothesised genetic founder event for blue eye colour occurring in northern Europe, leading to the blue eye colour phenotype and its particular distribution pattern as observed across Europe today [1, 2]. Comparison of panel A of Figure 2 with Figure 1 also shows a similarity between the phenotypic brown and blue eye colour distribution and the distribution of the TT and CC genotypes, respectively. At the individual level, the CC genotype corresponds closely with the blue eye colour phenotype as expected. Of the 1664 individuals carrying the CC genotype, 1554 (93.4%) have blue eyes. Conversely, the TT but also the CT genotypes are more strongly associated with brown eye colour. It appears that the presence of only one brown ancestral T allele is sufficient to produce a brown eye colour phenotype. Of the 742 individuals with the TT genotype, 728 (98%) have brown eyes, and of the 1434 individuals with the CT genotype, 1082 (75.5%) have brown eyes. Overall, of the 2176 individuals carrying the T allele either as homozygote or heterozygote state, 1810 (83%) have brown eyes. Hence, our dataset from across Europe confirms the importance of *HERC2* rs12913832 in the determination of blue and brown eye colours [6-7]. Furthermore, within the EUREYE dataset, we noticed that the presence of a homozygote TT at rs12913832 is more generally associated with a darker brown eye colour while a heterozygote genotype is more associated with a lighter brown eye colour (or hazel). Particularly, of the 742 rs12913832-TT carriers, 578 (77.9%) have dark brown eyes and 150 (20.2%) have light brown eyes, whereas of the 1434 rs12913832 CT carriers 527 (36.8%) have dark brown eyes and 555 (38.7%) have light brown eyes.

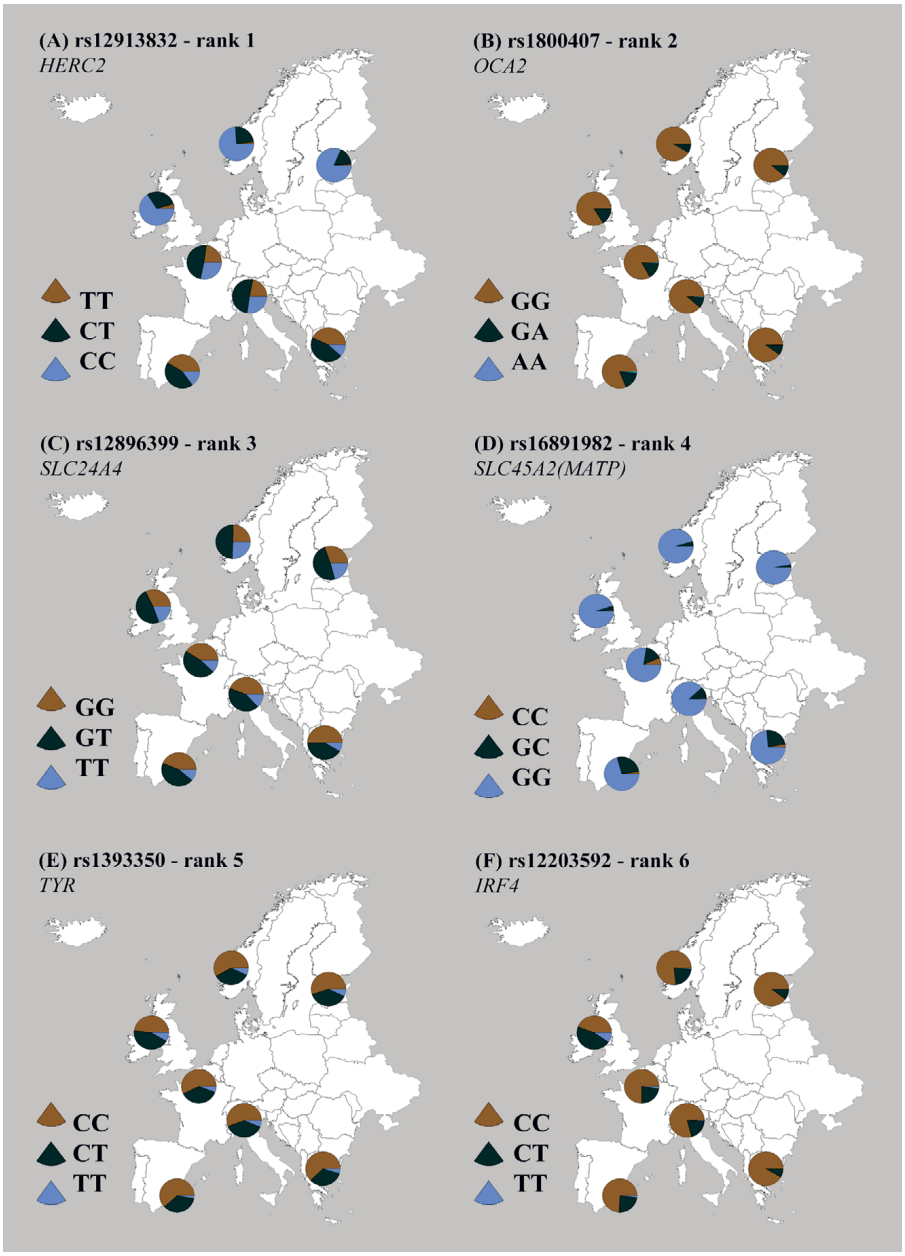

Figure 2: Genotype distribution of the six IrisPlex SNPs across seven European populations (see Legend of Figure 1 for the sampling locations). The respective homozygote allele associated with brown eye colour is depicted in brown, the respective homozygote allele associated to blue eye colour is depicted in blue, and black denotes the heterozygote stage. Rank indicates the eye colour prediction rank from a previous analysis considering 37 SNPs from 8 pigmentation genes [9] with 1 meaning most predictive. The gene names where the SNPs are located are also provided.

There is a statistically significant relationship (χ^2 = 171.9, df = 1, p = < 0.0001) between the number of rs12913832 T alleles and the degree of brown eye colour intensity, the incidence of darker brown is increased with TT alleles in comparison to CT alleles where there are a higher frequency of light brown/hazel eyes. These data are in line with the idea that the rs12913832C allele may be involved in partial haploinsufficiency in (eye-colour) pigment production [11].

As previously demonstrated [16], each of the 5 additional SNPs within the IrisPlex set has a smaller additive eye colour prediction effect on the model after the top ranked SNP rs12913832 from *HERC2*. The *OCA2* SNP rs1800407 (rank 2 in the overall prediction analysis [9]) does not show a strong genotype-phenotype correlation across Europe due to the very low frequency of its minor A allele. The A-allele was observed in 490 (12.8%) individuals in its heterozygous form and only 21 (0.5%) individuals in its homozygous form (Figure 2B). However, rs1800407 has previously been shown to contribute independently, albeit to a lesser degree, to eye colour variation [10]. Mutations in the *OCA2* gene result in several forms of oculocutaneous albinism causing a lack of pigmentation in the iris [25]. The non-synonymous mutation (A allele) of the rs1800407 SNP has also been previously associated with green/hazel iris colour [26]. The remaining SNPs within the IrisPlex, rs12896399 *SLC24A4* (Figure 2C), rs16891982 *SLC45A2* (Figure 2D), rs1393350 *TYR* (Figure 2E), and rs12203592 *IRF4* (Figure 2F) have smaller additive effects on the eye colour phenotype prediction determined by the rs12913832 *HERC2* genotype outcome as previously published [16], and do not show clear genotype-phenotype similarities to such a high degree as rs12913832 *HERC2* does. Interestingly however, Northern Ireland displayed a higher incidence

(10%) of the TT genotype of rs12203592 *IRF4* (rank 6 in the overall prediction analysis [9]) in comparison to the rest of Europe (1%). This particular SNP has previously been associated with Celtic ancestry and displays allele frequency versus ancestry gradients within the British Isles (comparing England with Scotland, Ireland and Wales) [27]. The known pigmentation function of *IRF4* may suggest a possible role for this SNP in controlling the particular lighter pigmentation phenotype (pale skin, red hair, blue/green eyes) typical for people of Celtic ancestry.

Assessing IrisPlex eye colour prediction probability threshold

Across all samples and phenotypes investigated here, the IrisPlex system with model parameters based on a previous single population study [9] performed with an overall accuracy of 87.5% in the 3840 individual predictions, varying between 83% (Italy) and 95.5% (Greece) across the seven European populations tested. However, the overall prediction error rate of 12.5% does not take any prediction probability threshold into account. In cases where the three probabilities are relatively similar to each other and the probability for the selected category is only marginally higher, there is an increased risk of an incorrect prediction being made. This leads to a considerably higher error rate being observed in such cases than if one of the three probabilities greatly exceeds the two others. In these instances it may be prudent to apply a probability threshold to provide a higher accuracy of prediction for blue and brown eye colour to the model as a whole, especially for use in forensic casework. Applying a probability threshold for IrisPlex eye colour prediction, where the highest of the three probabilities must be higher than a defined limit, overcomes this chance of error.

Although this was briefly described in our previous publication [16], the large amount of new genotype and phenotype data within the present study allowed a more substantial investigation into the threshold definition. To find the ideal threshold for the model as a whole, we examined the levels of accuracy and precision of prediction calls at varying thresholds, from p≥0.5 to p≥ 0.95, while taking into account the cost (in terms of numbers of samples unpredictable as the highest p value does not reach the respective threshold) to the total set. For each individual in the EUREYE set the probabilities of having blue, brown and intermediate eye colours, given their specific 6 SNP genotypes, were generated, with the highest probability obtained

Threshold (*p*) cut-off	Correct calls	Incorrect calls	Prediction error of total samples ≥ threshold (%)	Number of samples ≥ threshold	Loss of samples < threshold (%)
≥0	3362	478	12.5	3840	0
≥0.5	3221	343	9.62	3564	7.20
≥0.55	3158	312	9.0	3470	9.60
≥0.6	3083	272	8.1	3355	12.60
≥0.65	2904	211	6.8	3115	18.90
≥0.7	2793	182	6.1	2975	22.50
≥0.75	2601	139	5.1	2740	28.60
≥0.8	2406	112	4.45	2518	34.40
≥0.85	2314	107	4.42	2421	36.90
≥0.9	1811	48	2.6	1859	51.60
≥0.95	904	9	1.0	913	76.22

Table 2: Accuracy of initial 'IrisPlex' model combining categories blue, brown and undefined at varying test thresholds. Data includes correct and incorrect eye colour predictions in relation to phenotypic images; the percentage error and the percentage loss of samples or samples that are deemed undefined at the varying probability (p) threshold levels from ≥0.5 to ≥0.95 highest eye colour prediction value.

determining the final predicted eye colour. Prediction thresholds, ranging from p≥0.5 to p≥0.95 were then used to assess the obtained probability – if an individual returned a p value below the given threshold, they were reclassified as 'undefined'. The number of individuals with correctly predicted phenotypes, and the number that could not be predicted, were then compared for each threshold level (Table 2). Using the extreme thresholds tested as illustrative examples; we show that a low threshold of p≥0.5 resulted in 3221 (90.38%) of the total 3564 individuals tested being predicted correctly above that set threshold, with 343 (9.62%) predicted incorrectly. From the entire EUREYE set, 276 (7.2%) were deemed undefined as they fell below the set threshold and were unpredictable at this threshold. Using a high threshold of p≥0.95, 904 (99%) of the total 913 individuals above that set threshold were predicted correctly and only 9 (1%) were incorrectly predicted; however, the loss to the entire EUREYE set was extreme, as 2927 out of 3840 individuals (76.22%) were unpredictable below this p≥0.95 threshold and were classified into the undefined category (Table 2).

Hence, for defining the most appropriate threshold we believe an acceptable level need to be found between three aspects: i) the numbers of predictable individuals above the threshold, ii) the numbers of correctly predicted individuals, and iii) the numbers of incorrectly predicted ones. By applying a threshold of p≥0.7, 2793 of 2975 (93.9%) individuals above that set threshold were correctly predicted with only 182 (6.1 %) incorrect calls. This probability threshold gave the most acceptable results with a very high accuracy level of 93.9% (nearest to 95% confidence), and a loss of only 865 samples from the total 3840 EUREYE set (22.5%) deemed as undefined. This 22.5% of individuals make up the undefined category and

they contain prediction probabilities that do not allow them to be assigned to the true blue or true brown eye colour categories. These include individuals with heterochromia (i.e., ring around pupil of different colour), as well as those with intermediate colours such as green. However, it also includes phenotypes that were correctly predicted, but assigned a lower probability than the defined set threshold. This may be due to a particular genotype combination observed, where some European light brown-eyed (hazel) individuals possess the derived blue-associated alleles for the lower ranked SNPs which lower brown predicted p values. Across all threshold levels examined however, we believe an eye colour prediction probability threshold of $p \geq 0.7$ accounts for an acceptable error rate and percentage loss of samples, thus maximising as much as possible the potential of the IrisPlex system. Figure 3 provides the total correct, total incorrect and total undefined category numbers of the EUREYE set applying a prediction probability threshold of $p \geq 0.7$ for all seven European populations. As evident the average number of correct calls across Europe was 71% of the total EUREYE set, ranging from 62% in North Italy to 80% in Greece, whereas the average number of incorrect calls across Europe was 6% of the total EUREYE set, ranging from 2% in Greece to 8% in Estonia. The average nmber of individuals that made up the undefined category across Europe was 23% of the total EUREYE set when using the $p \geq 0.7$ threshold, ranging from 13% in Estonia to 33% in Italy.

However, IrisPlex users are free to define their own threshold. Notably, prediction probabilities of p≥0.5 still have a highly accurate call rate (90.4%), while maximising the number of samples from the total set (loss of only 7.2% of the total set at p≥0.5 threshold) at which a phenotype prediction can be obtained. In particular, prediction probabilities between 0.5 and 0.7 can indicate intermediate-blue (such as blue-green) or intermediate-brown (such as yellow-hazel) phenotypes, due to the mainly additive nature of the SNP model. We noticed that the prediction error for brown when applying the lower thresholds (0.5 - 0.7) was slightly higher than it was for blue (Table 3). This may be influenced by the observed presence of derived

Threshold (*p*) cut-off	Blue correct calls	Blue incorrect calls	Blue prediction error (%)	Brown correct calls	Brown incorrect calls	Brown prediction error (%)
≥0	1591	172	9.8	1763	289	14.1
≥0.5	1559	118	7.0	1662	224	11.9
≥0.55	1554	112	6.7	1604	199	11.0
≥0.6	1553	109	6.6	1530	163	9.6
≥0.65	1552	109	6.6	1352	102	7.0
≥0.7	1544	102	6.2	1249	81	6.1
≥0.75	1528	96	5.9	1072	43	3.9
≥0.8	1504	92	5.8	902	20	2.2
≥0.85	1473	91	5.8	841	16	1.9
≥0.9	1203	39	3.1	698	8	1.1
≥0.95	381	5	1.3	523	4	0.8

Table 3: Blue and brown eye colour prediction accuracy, including correct calls, incorrect calls and error rate, separated by category and threshold limits of ≥0.5 to ≥0.95 highest eye colour prediction probability (p) value from 3840 EUREYE individuals of seven populations from various parts of Europe.

blue-associated alleles at some of the SNPs in some brown-eyed Europeans. Notably, brown eye colour prediction probabilities for brown-eyed Europeans are often lower than those for non-Europeans, who are usually of brown eye colour (levels of over 0.99 p have been observed outside Europe [16]) because the derived blue SNP alleles are usually not present in people of non-European ancestry due to the origin and selective history of non-brown eye colour in Europe [16].

IrisPlex prediction model parameters from across European data

The initial IrisPlex prediction model as published earlier [9, 16-17] was generated from eye colour genotype and phenotype data from one, albeit large, European population set of 3804 individuals from a total of 6168 with European ancestry from The Netherlands. To investigate whether using pan-European data provides more accurate model parameters for eye colour prediction than previously obtained from a single European population, the EUREYE data set was used to generate a new prediction model (referred to as the 'EUREYE IrisPlex' model). For this purpose, the overall EUREYE dataset was randomly split into a model-building dataset of 80% of samples (N = 3071) and a model verification set of 20% (N = 769) to allow assessment of the specificity and sensitivity of the model's prediction power. Using the multinomial logistic regression approach, α values testing the 3 categories blue, brown and intermediate colour, and β parameters for each of the 6 SNPs and their contribution to each category were generated from the model-building dataset, allowing the production of a new EUREYE IrisPlex model with our previously published formula [9].

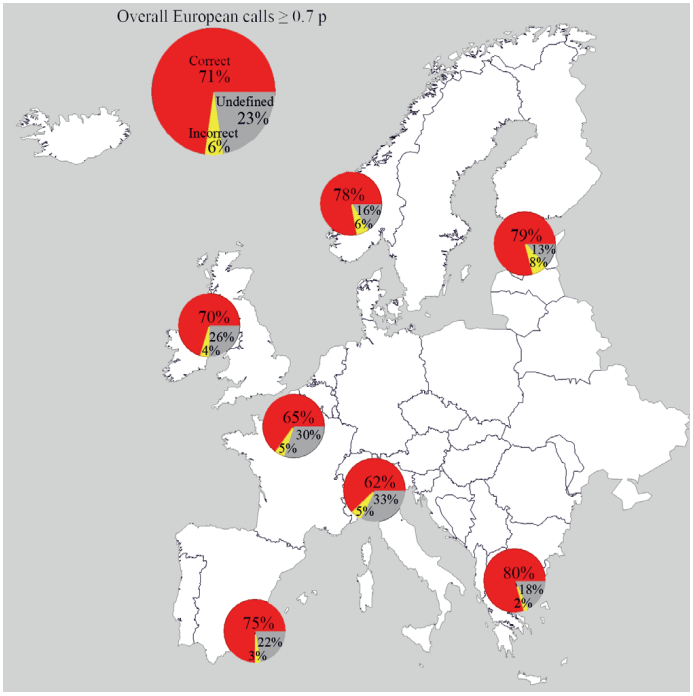

Figure 3: Graphical representation of the call categories of the 'IrisPlex' model within each of the seven European populations (see Legend of Figure 1 for the sampling locations) at the p≥0.7 threshold level per population and overall. Red indicates the correct number of eye colour predictions; yellow indicates the number of incorrect eye colour predictions, and the light grey indicates samples deemed 'undefined' and unpredictable in respect of eye colour.

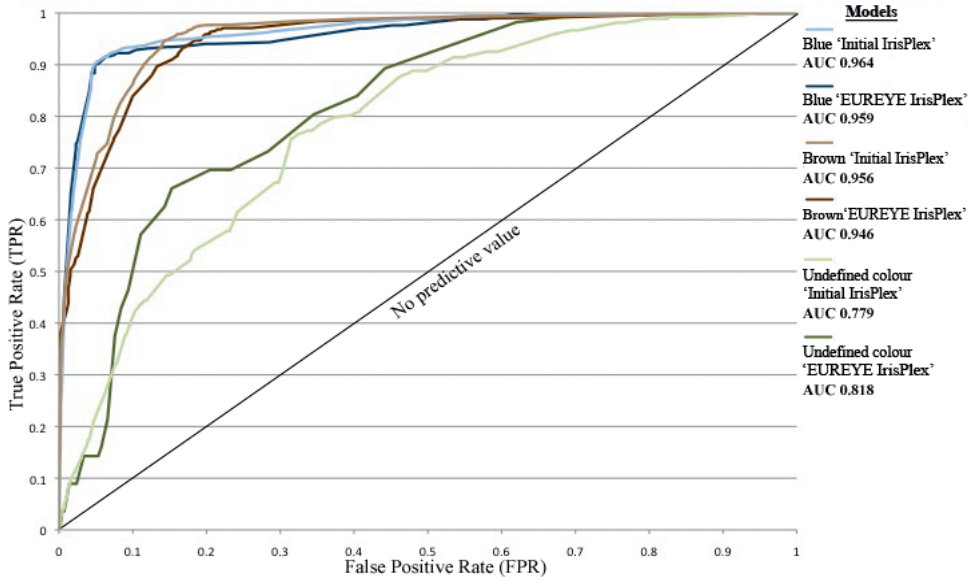

Figure 4: ROC curve analysis of both models: 'EUREYE IrisPlex' model and the 'Initial IrisPlex' model plotting False Positive Rate vs True Positive Rate for each eye colour category. Each category of blue, brown and intermediate eye colour is compared for accuracy and an AUC measurement is stated. The 'EUREYE IrisPlex' model is based on 3071 (80%) EUREYE individuals, which represent the model building dataset, and 769 (20%) EUREYE individuals representing the model verification dataset. The 'Initial IrisPlex' model is based on predicting all 3840 (100%) EUREYE individuals.

The model-verification dataset was then analysed for eye colour prediction into the blue, brown or intermediate colour categories for each individual. A ROC (receiver operating characteristic) curve, was generated, and the results were compared to those obtained from analysing the entire set of 3840 EUREYE individuals under the previously published [9, 16] IrisPlex model (referred to as the 'Initial IrisPlex' model), (Figure 4). Overall, no statistically significant difference was observed in the AUC values for the three eye colour categories derived from the Initial IrisPlex model, and the EUREYE IrisPlex model (Mann Whitney U Test U= 4, p = 0.827), demonstrating that both models yield the same eye colour prediction accuracies.

That the Initial IrisPlex model, which was based on a single European population, behaves in a similar fashion as the EUREYE IrisPlex model, which is based on seven populations from across Europe differing in eye colour frequencies, clearly demonstrates that our initial model was not biased by the use of a single European population. It should also be underlined that the extremely high predictive power of 0.96 AUC for blue and 0.96 AUC for brown as established in the EUREYE dataset, are higher than those previously reported from Dutch Europeans of the Rotterdam Study at 0.925 for brown and 0.91 for blue using the same SNPs [9]. Factors that potentially contributed to the increased IrisPlex prediction accuracy seen in the EUREYE data are better eye image quality (we included pupils that were undilated and therefore the entire iris could be seen), multiple images at different vantage points per individual gave easier individual categorisation, and possibly the use of a single grader approach on the digital images as opposed to the ad-hoc approach where differing light conditions may affect grading.

Practical use of the IrisPlex system

Included in our initial IrisPlex article [16] we made an excel spreadsheet available as online supplementary material to obtain eye colour probabilities from IrisPlex genotypes. Since this current study showed that the prediction parameters obtained with the EUREYE IrisPlex model are very similar to those previously obtained with the Initial IrisPlex model, we see no need to update this spreadsheet. Hence, we refer the IrisPlex user to the Supplementary Table 4 of our previous paper [16] for obtaining eye colour probabilities from IrisPlex genotypes. As a guideline, the highest probability from the three categories blue, brown or intermediate shall reflect the predicted eye colour as long as one of the three probabilities exceed the other two. As outlined above, the practical use of DNA-based eye colour prediction probabilities depends on the actual probability value estimated with more strength given to values above a defined threshold and less strength to values below. Although in this study we found a threshold of $p \geq 0.7$ most suitable, many individuals with single probabilities below this threshold were also correctly predicted. The information provided in Table 2 and 3 of the current study will be useful as a guide to thresholds and error rates based on data from over 3000 Europeans collected at seven sites (EUREYE) and is useful in assessing the strength of IrisPlex eye colour prediction. For instance, eye colour prediction probabilities over 0.95 are correct in 99% of cases using this dataset, and could be reported in such a way. If, however, all three eye colour prediction probabilities turn out to be similar, IrisPlex is not informative for eye colour prediction and the results should be reported as such.

Other SNPs for eye colour prediction?

In one of our earlier studies [9], we tested all at the time available eye colour associated SNPs for their predictive effect on eye colour and showed that the obtained prediction accuracies were considerable higher than those reported in previous studies. Subsequently, we used the six SNPs that covered almost all of the prediction accuracy achieved in the study [9] for IrisPlex development [16]. To keep up to date on recent research, we now ask the question, are there currently more informative SNPs for eye colour model prediction than those included in the IrisPlex system? Branicki et al. [10] recommend the use of rs12913832 *HERC2* and rs1800407 *OCA2* for eye colour prediction, both these SNPs are included in IrisPlex (rank 1 and 2, respectively). Mengel-From et al. [12] reported that four SNPs reach a 29.3 likelihood ratio in discriminating between light and dark eye colour in Europeans of which two, rs12913832 *HERC2* and rs1800407 *OCA2,* are included in IrisPlex (rank 1 and 2, respectively). The other two SNPs, rs1129038 *HERC2* and rs11636232 *HERC2*, are known to be in strong linkage disequilibrium with the most eye-colour predictive IrisPlex SNP rs12913832 *HERC2*; it therefore is unlikely that their inclusion would increase eye colour prediction accuracies achieved using IrisPlex. Liu et al. [15] identified three new genomic regions for eye colour when testing for association with quantitative measures of eye coloration extracted from digital eye images of people of European ancestry. However, a model including these new eye colour SNPs together with previously known eye colour SNPs provided AUC values for blue at 0.92 and brown at 0.93, which are only marginally larger than those achieved previously with the IrisPlex system [9], but are considerably lower than those achieved here with IrisPlex using the EUREYE data. Recently,

Pospiech et al. [28] used 24 pigmentation SNPs, including all IrisPlex SNPs, to test gene-gene interaction effects on eye colour in a Polish sample. One notable additional SNP is rs1408799 in *TRYP1* for which an interactive effect together with the IrisPlex SNPs rs12913832 *HERC2* and rs1800407 *OCA2* for green versus non-green eye colour was reported. However, no independent effect of rs1408799 was found and future work may show how much this SNP actually contributes to green eye colour and its prediction.

Two additional studies pooled individuals from various bio-geographic ancestries for ascertaining SNPs to predict eye colour. Valenzuela et al. [29] reported on three SNPs that explained 76% of eye colour variation, of which two, rs12913832 *HERC2* and rs16891982 *SLC45A2*, are included in IrisPlex (rank 1 and 4, respectively) whereas the third SNP, rs1426654 *SLC24A5*, is not. They also reported rs1426654 to be informative in the explanation of hair melanin content in the same individuals [29]. We recently demonstrated that it is unlikely that rs1426654 *SLC24A5* truly contributes to hair colour variation as this SNP was only seen in its heterozygote state in 1.3% of a Polish population sample expressing large variation of hair colours (the 5 heterozygote carriers also belonged to three different hair colour categories), while 98.7% of the individuals carried the homozygote derived allele AA [30]. Because of this finding, together with the strong relationship between hair and eye colour variation, it is unlikely that rs1426654 *SLC24A5* contributes to eye colour variation. Additional support comes from previous studies showing that the A-allele exists in very high frequencies in various European populations with large variation in hair and eye colour variation [31, 32]. It therefore is likely that the rs1426654 findings of

Valenzuela et al. [29] in respect of hair melanin and eye colour reflect bio-geographic ancestry effects rather than genuine pigmentation effects. In the other multi-ethnic study, Spichenok et al. [33] reported six SNPs for eye colour prediction of which three, rs12913832 *HERC2*, rs16891982 *SLC45A2*, and rs12203592 *IRF4* are included in IrisPlex (rank 1, 4, and 6, respectively), whereas three others rs1545397 *OCA2*, rs6119471 *ASIP*, and rs885479 *MC1R* are not. Rs1545397 *OCA2* shows allelic variation in Europeans with the major allele being the ancestral one, but also in Asians with the derived allele being the major one [33]; however, Asians do not carry variation in eye colour so that it is unlikely that rs1545397 is a good predictor of eye colour. Rs6119471 near *ASIP* shows no allelic variation in Europeans and Asians but does show variation in the Africans [33], which contradicts knowledge on global distribution of eye colour variation, also making rs6119471 an unlikely predictor of eye colour. Rs885479 is in *MC1R* and this gene has been identified as a major hair and skin colour gene [5, 8, 30]; however, p-values of *MC1R* SNPs for eye colour association do not provide convincing evidence for direct involvement [5], although an interaction effect of SNPs in *MC1R* with other pigmentation SNPs, including some in IrisPlex, has been reported [10, 28]. Rs885479 is almost fixed in Africans, shows considerably low variation in Europeans but displays high variation in Asians [33]. As this is inconsistent in relation to knowledge on the global distribution of eye colour variation, it makes it unlikely that rs885479 is a good predictor of eye colour. The continental allele frequency distributions together with knowledge on the global distribution of eye colour variation suggest that the apparent eye colour effect reported by Spichenok et al. [33] for the three non-IrisPlex SNPs rs1545397, rs6119471 and rs885479 may rather be an effect of bio-

geographic ancestry. Notably, these three SNPs are claimed by Spichenok et al. [33] to be informative for brown eye colour, which is the expected eye colour when effects are dampened with non-European bio-geographic ancestry. These two multi-ethnic studies [29, 33] demonstrate the limitations in combining data of individuals with various continental ancestries for developing eye (and hair) colour predictive SNPs, as the effect of bio-geographic ancestry simply cannot be separated from genuine effects of eye (and hair) colour under such study design. However, once eye (and hair) colour predicting SNPs have been developed from European data, they should be applied to non-European populations for validation purposes as we suggested and executed previously [16].

CONCLUSIONS

This study provides convincing empirical proof that the IrisPlex model works reliably for accurate prediction of blue and brown eye colour. Together with the recently published forensic developmental validation of the IrisPlex assay [17], the IrisPlex system, including evidence provided here from data across Europe, is highly informative and suitable for predicting blue and brown eye colour in forensic applications. Further work is needed to increase the level of detail on which DNA-based eye colour prediction can be achieved (including the intermediate eye colours such as green), if possible moving from categorical eye colour prediction towards DNA prediction of various eye colour shades. The latter could result in providing a more realistic eye colour description such as colour chart or colour print out predicted from DNA instead of a broad eye colour category as is currently possible. Such a detailed approach should reduce

the potential problems caused by perceptual differences between observers that may occur in the practical application of DNA-based categorical eye colour prediction in police intelligence investigations. In the future, DNA-based eye colour prediction may be combined with DNA-based hair colour prediction [30] and DNA-based age group estimation [34], as well as with additional EVCs that may become predictable from DNA, to further describe the appearance of an unknown perpetrator, which will be useful for concentrating police investigations in suspect-less cases that usually cannot be solved via standard forensic STR profiling.

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Supplementary Material can be found at

http://www.sciencedirect.com/science/article/pii/S187249731100144X

Or the **APPENDICES** of this Thesis.

CHAPTER 2.4

A NEW APPROACH FOR QUANTIFYING HUMAN PIGMENTATION FROM DIGITAL IMAGERY: IMPROVED EYE COLOUR PHENOTYPING ENHANCES GENETIC ASSOCIATION AND PREDICTION

Manuscript in Preparation

CHAPTER 3

DNA PHENOTYPING: HAIR COLOUR PREDICTION

CHAPTER 3.1

The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA.

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ABSTRACT

Recently, the field of predicting phenotypes of externally visible characteristics (EVC's) from DNA genotypes with the final aim of concentrating police investigations to find persons completely unknown to investigating authorities, also referred to as Forensic DNA Phenotyping (FDP), has started to become established in forensic biology. We previously developed and forensically validated the IrisPlex system for accurate prediction of blue and brown eye colour from DNA, and recently showed that all major hair colour categories are predictable from carefully selected DNA markers. Here, we introduce the newly developed HIrisPlex system, which is capable of simultaneously predicting both hair and eye colour from DNA. HIrisPlex consists of a single multiplex assay targeting 24 eye and hair colour predictive DNA variants including all 6 IrisPlex SNPs, as well as two prediction models, a newly developed model for hair colour categories and shade, and the previously developed IrisPlex model for eye colour. The HIrisPlex assay was designed to cope with low amounts of template DNA, as well as degraded DNA, and preliminary sensitivity testing revealed full DNA profiles down to 63 pg input DNA. The power of the HIrisPlex system to predict hair colour was assessed in 1551 individuals from three different parts of Europe showing different hair colour frequencies. Using a 20% subset of individuals, while 80% were used for model building, the individual-based prediction accuracies employing a prediction-guided approach were 69.5% for blond, 78.5% for brown, 80% for red and 87.5% for black hair colour on average. Results from HIrisPlex analysis on worldwide DNA samples imply that HIrisPlex hair colour prediction is reliable independent of bio-geographic ancestry (similar to previous IrisPlex findings for eye colour). We furthermore

demonstrate that it is possible to infer with a prediction accuracy of >86% if a brown-eyed, black-haired individual is of non-European (excluding regions nearby Europe) versus European (including nearby regions) bio-geographic origin solely from the strength of HIrisPlex eye and hair colour probabilities, which can provide extra intelligence for future forensic applications. The HIrisPlex system introduced here, including a single multiplex test assay, an interactive tool and prediction guide, and recommendations for reporting final outcomes, represents the first tool for simultaneously establishing categorical eye and hair colour of a person from DNA. The practical forensic application of the HIrisPlex system is expected to benefit cases where other avenues of investigation, including STR profiling, provide no leads on who the unknown crime scene sample donor or the unknown missing person might be.

INTRODUCTION

Over the last few years, the prediction of externally visible characteristics (EVCs) from DNA has been an interesting topic of study for many reasons, in particular, its anticipated use within forensic genetics [1-3] resulting in the chosen term Forensic DNA Phenotyping (FDP). The ability to predict the physical appearance of an individual directly from crime scene material can in principle help police investigations by limiting a large number of potential suspects in cases where perpetrators unknown to the investigating authorities are involved. These include cases where conventional STR profiling could not provide a hit within the forensic DNA (profile) database, or could not provide a match with a suspect singled-out by police investigation, or cases where an STR profile could simply not be generated due to low quality and/or quantity of DNA available. Using EVC information obtained from the crime scene material via FDP, police would then proceed with more concentrated enquires, and finally request standard forensic STR profiling only for the reduced number of EVC matching suspects aiming DNA individualisation for court room use. Obviously, the more EVC's that are predictable from crime scene material, the better a person's appearance can be described, and in turn the smaller the number of appearance-matching potential suspects for subsequent forensic STR profiling. Also in missing person cases where a body was found decomposed with no EVC information discernable from visual inspection, or body parts that do not provide EVC information including bones, FDP is expected to provide leads for finding the right antemortem samples or family members for final STR-based identification.

The use of DNA (or other biomarkers) for investigative purposes termed 'DNA intelligence', rather than for identification purposes in the

court room as currently applied in forensics, marks a completely new application of DNA in forensics and is currently at the early stages of development. At present there is only one FDP tool available that has already been developmentally validated for forensic use and that is the IrisPlex system, capable of predicting eye colour from DNA [4, 5]. Although other studies have suggested DNA markers and methods for predicting externally visible traits, most notably eye colour [6-18]none of them introduced a tool that had undergone systematic forensic developmental validation testing as of yet. The IrisPlex system allows the prediction of eye colour from minute amounts of DNA (31pg DNA input full profiles) and has proven to be 94% accurate for predicting blue and brown eye colour when tested on a European set of >3800 individuals [19]. However, work is on-going with regards to identifying the underlying genes and developing predictive DNA markers for several other EVCs [3] such as skin colour [8, 20, 21], hair colour [6, 8], body height [22] [23], male baldness [24], and hair morphology [25, 26].

The previous progress on categorical eye colour DNA predictability together with the strong genetic and phenotypic relationship between eye and hair colour variation, as well as the increased understanding of the genetic basis of hair colour, all suggest that hair colour may represent the next-promising candidate EVC for DNA prediction after eye colour. Hair colour (as well as eye colour), is generally known to be highly variable in people of (at least partial) European descent and those from nearby regions such as the Middle East and parts of Western Asia [27], with individuals displaying numerous variations of hair colour shade that are usually summarised in four main categories of colour such as red, blond, brown and black. In contrast, people from any

other parts of the world (and without European / nearby genetic admixture) usually display the ancestral black hair colour (together with the ancestral brown eye colour) phenotype. Variation in hair (and eye) colour is assumed to be of European origin and is thought to have reached their currently observed frequencies via sexual selection (i.e., mate choice preferences) [28]. The genetic basis of human hair colour variation has been studied considerably in the last few years. Recent studies either employing the candidate gene approach or genome-wide association and/or linkage analysis have identified genes and DNA variants likely to be involved in human hair colour variation [6-8, 12, 14, 29-33]. Some preliminary attempts have already been made towards the prediction of hair colour from informative DNA variants. In fact, an early red hair prediction protocol based on a combination of non-synonymous single nucleotide polymorphisms (SNPs) in the *MC1R* gene that incur the red hair phenotype effect was already developed for forensic use more than ten years ago [34] and its accuracy was 84% in the prediction of red hair individuals. Sulem et al. [6] in their genome-wide association study for European pigmentation traits developed a hair colour prediction tool, which was capable of excluding red and either blond or brown hair colour in its prediction for many of their individuals. More recently, Valenzuela et al [16] assessed 75 SNPs from 24 genes previously implicated in hair, skin and eye colour in samples of various bio-geographic origins (Europe and elsewhere) and found that three of them i.e., rs12913832 (*HERC2*), rs16891982 (*SLC45A2*) and rs1426654 (*SLC24A5*) combined gave the best prediction for light and dark hair colour.

Armed with previous knowledge on hair colour associated DNA variants and in considering the most up-to-date list of DNA variants

related to human hair colour variation available at the time, we recently performed an evaluation of 46 SNPs from 13 genes [35] for model-based population-wise hair colour prediction aiming to find a set of most hair colour predictive DNA variants. In this previous study we identified a set of 13 DNA markers (2 *MC1R* combined marker sets and 11 single DNA markers) from 11 genes (*MC1R, HERC2, OCA2, SLC45A2 (MATP), KITLG, EXOC2, TYR, SLC24A4, IRF4, PIGU/ASIP and TYRP1*) containing most hair colour predictive information. This DNA marker set provided a high degree of population-based, prevalence-adjusted overall prediction accuracy as expressed by the area under the curve of a receiver operating characteristic curve (AUC) with estimates at 0.93 for red, 0.87 for black, 0.82 for brown, and 0.81 for blond hair colour, where 1 means completely accurate prediction. However, the genotyping methodology used in this previous screening study did not allow simultaneous genotyping of all 22 identified hair colour predictive DNA markers in a single reaction as would be appreciated in forensic DNA analysis where there can be limited amounts of starting material. Furthermore, in the previous study, only samples with hair colour genotypes and phenotypes from a single country in Eastern Europe i.e., Poland, were available, whereas the inclusion of individuals from other European regions, such as Western and Southern parts, would be beneficial in order to enrich with individuals displaying hair colours such as brown and black that are more common in these parts of Europe.

In the present study, we developed and evaluated the sensitivity of a single-tube multiplex assay targeting the 22 previously recognised hair colour predictive DNA variants as well as the six eye colour predictive SNPs from our previously developed IrisPlex system (four of which are

overlapping), we employed the SNaPshot technology because it can be easily implemented in forensic DNA laboratories as no additional equipment or serious interference with protocols is needed to apply it. Furthermore, we assessed the power of the 22 DNA variants to predict hair colour categories, as well as shade, via model-based prediction studies using an expanded database of hair colour genotype and phenotype data for >1500 individuals from Eastern, Western and Southern parts of Europe that displayed varying degrees of hair colouration. Moreover, we investigated via analysing a worldwide set of individuals from 51 populations (HGDP-CEPH), whether or not the reliability of hair colour prediction available with these 22 DNA variants depends on knowledge of bio-geographic ancestry. We present and make available for future use, the first system for parallel prediction of hair and eye colour from DNA we termed HIrisPlex, consisting of a single multiplex assay for 24 eye and/or hair colour predictive DNA variants and two prediction models i.e. a newly developed model for hair colour and shade prediction and the previously developed IrisPlex model for eye colour prediction. An interactive spreadsheet tool for obtaining individual hair colour, hair colour shade, and eye colour prediction probabilities from HIrisPlex genotypes as well as a prediction guide for accurate interpretation of individual hair colour and shade probabilities are made available to enhance the practical use of the HIrisPlex system in future applications such as forensics.

MATERIALS & METHODS

Subjects, imagery and hair and eye colour classification

DNA samples and hair colour information was collected from 1551 European subjects living in Poland (n=1093), the Republic of Ireland (n=339) and Greece (n=119). All participants gave informed consent. The study was approved in part by the Ethics Committee of the Jagiellonian University, number KBET/17/B/2005 and the Commission on Bioethics of the Regional Board of Medical Doctors in Krakow number 48 KBL/OIL/2008. Hair and eye colour phenotypes were collected by a combination of self-assessment and professional single observer grading (Polish data). The professional grader (AKK) for the polish dataset is a medical doctor (dermatologist) who evaluated hair colour upon observation, and questioning of individuals in circumstances where hair was dyed or grey. For hair colour phenotype self-assessment, individuals were asked to fill into the questionnaire, the colour of their hair during their 20's, and at what age grey/white hairs started to appear (Irish collection), this avoided the effects of hair greying and whitening on phenotyping. Sample collection in Ireland included high-resolution eye and hair photographic imagery. In a brief description, hair and eye images were taken using a Nikon D3100 with an AF-S Micro Nikkor 60mm macro lens, the aperture, shutterspeed and ISO were fixed to f=22, 1/125, and 200 respectively. A ring flash (model Speedlight SB-R200) and an average distance of 7cm was used from the eye and from the back of the head for hair imagery. This ensured consistent sampling and regulated lighting conditions, including lens settings of a 0.2 and 0.23 fixed focal length. All individuals were asked to fill in a questionnaire that included basic information, such as gender and age as well as data concerning eye and

hair pigmentation phenotype. However, due to many Irish individuals having dyed or grey hair, self-reported hair colour classifications were used for this set in model training. For the Greek collection, a buccal swab was taken from each individual and a self-reported questionnaire regarding hair and eye colour information was collected. For both the Irish and Greek set, hair colour was classified into 7 categories: blond (5.9%), light-brown (34%), dark-brown (45.2%), auburn (5.7%), blond-red (1.3%), red (2.2%), and black (5.7%). For the Polish dataset, this data was collected as previously reported [35] and hair colour was classified into 7 categories: blond (13.7%), dark-blond (44.2%), brown (22.6%), auburn (1%), blond-red (3.9%), red (3.8%), and black (10.8%)). For hair colour prediction analyses, we grouped blond and dark-blond into one blond category (42.6%), light brown and dark brown into one brown category (39.3%) and auburn, blond-red, and red into one red category (8.8%) with black as an additional fourth category (9.3%). Eye

Hair colour	Blond	Dark blond*/light brown	Dark brown	Brown red/auburn	Blond red	Red	Black	Total	Eye colour – blue	Intermediate (green, heterochromia)	Brown	Total	Male	Female	Total
Poland	150	483*	247	11	43	41	118	1093	590	164	339	1093	449	644	1093
Ireland	16	111	158	23	6	10	15	339	172	90	77	339	77	262	339
Greece	11	45	49	3	0	0	11	119	13	15	91	119	51	68	119
Total	177	639	454	37	49	51	144	1551	775	269	507	1551	577	974	1551

* represents individuals who were reported as dark blond in the dark blond/light brown category.

Table 1: Phenotype frequencies according to hair and eye colour categories (including sex) for the full combined set of individuals from Poland, Ireland and Greece.

colour was classified into 3 categories blue, brown and intermediate (including green). The term category in this context refers to the grouping of similar phenotypic colours into one group to separate them from another colour group, i.e. blond category, black category. Table 1 displays the numbers of hair and eye colour phenotypes including sex, within all 3 populations sampled. Notably red hair in the Polish population and green eye colour in the Irish population were intentionally enriched due to their rare occurrence, therefore both phenotypes do not reflect natural population frequencies.

DNA samples and HIrisPlex genotyping

DNA from the Polish samples was extracted as described previously [35]. Saliva samples collected from individuals in Ireland were extracted using the Puregene DNA isolation kit (Qiagen, Hilden, Germany). Buccal swabs collected from individuals in Greece were extracted using an in-house organic extraction protocol. DNA from the H952 subset of the HGDP-CEPH panel that represents 952 individuals from 51 worldwide populations [36, 37] were purchased from CEPH. Due to lack of DNA in some samples belonging to the HGDP-CEPH 952 set, 7 individuals could not be genotyped by the HIrisPlex assay, and therefore the final number of worldwide samples was 945.

All samples were genotyped using the HIrisPlex assay. The assay includes 23 SNPs and 1 insertion/deletion (INDEL) polymorphism, altogether 24 DNA variants, from 11 genes: *MC1R, HERC2, OCA2, SLC24A4, SLC45A2, IRF4, EXOC2, TRYP1, TYR, KITLG,* and *PIGU/ASIP*. Further information on these 24 markers can be found in Table 2, including primer sequences. The 24 PCR primer pairs were designed using the default

parameters of the program Primer3Plus [38], which is a free web-based design software. PCR fragments were designed to be as short as possible to cater for degraded DNA, and therefore all are less than 160 bp in length. To reduce the possibility of primer pairs interacting with each other, the program Autodimer [39] was used to analyse primer sequences. Surrounding sequence regions were also searched with BLAST [40] against dbSNP [41] to reduce the chance of a primers location covering a known interfering SNP site for efficient primer binding.

For the population genotyping, genomic DNA quantities ranging from 300 pg-3 ng in 1 µl formats were amplified per individual in a 10 µl reaction volume consisting of 1X PCR buffer, 2.5 mM $MgCl_2$, 220 µM of each dNTP, and 1.75 U AmpliTaq Gold DNA polymerase (Applied Biosystems Inc., Foster City, CA) including PCR primer concentrations found in Table 2. Thermo-cycling was performed on the 96-well GeneAmp® PCR system 9700 (Applied Biosystems) under the following conditions (1) 95°C for 10 min, (2) 33 cycles of 95°C for 30s and 61°C for 30s, (3) 5 min at 61°C. PCR products were cleaned with ExoSAP-IT (USB Corp., Cleveland, OH), as recommended by the manufacturer. Following removal of unincorporated dNTPs and primers. The multiplex SBE (single base extension) assay was performed using 2 µl of product with 1 µl of ABI SNaPshot kit (Applied Biosystems, Foster City, CA) reaction mix in a total reaction volume of 5 µl. Single base extension (SBE) primer sequences and concentrations used in the assay can be found in Table 2. Thermocycling conditions were as follows: 96°C for 2 min and 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 30s. Products were cleaned using SAP (USB Corp.), following manufacturers guidelines and 1 µl of cleaned product

was run on the ABI 3130xl Genetic Analyser (Applied Biosystems) with POP-7 on a 36 cm capillary array following the SNaPshot kit sample preparation guidelines, however run parameters of 2.5kV for 10 s injection voltage and run time of 500 s at 60 °C were used for increased sensitivity.

For assay sensitivity studies, genotyping results from two different individuals were assessed from serial dilutions of DNA input samples of 500 pg, 250 pg, 125 pg, 63 pg and 31 pg. Each result was investigated for allelic drop out, which includes peaks below the 50-rfu threshold that cannot be called. The determination of sensitivity was based on the production of a full profile in every replicate at a particular DNA input level.

Assay position	SNP	CHR	Position		Gene	Major Allele	Minor Allele	PCR primers		Concentration		Product size	SBE primers	Concentration
1	N29insA	16	89985753	Exonic	*MC1R*	C	insA	MC1Rset1F	Set1	0.55 µm	GCAGGGATCCCAGAGAAGAC	117bp	CCCCAGCTGGGGCTGGCTGCCAA	1.3 µm
2	rs11547464	16	89986091	Exonic	*MC1R*	G	A	MC1Rset1R		0.55 µm	TCAGAGATGGACACCTCCAG		ttttttttttttGCCATCGCCGTGGACC	0.1 µm
3	rs885479	16	89986154	Exonic	*MC1R*	C	T	MC1Rset2F	Set2	0.5 µm	CTGGTGAGCTTGGTGGAGA	158bp	ttttttttttttttttttGATGGCCGCAACGGCT	1.25 µm
4	rs1805008	16	89986144	Exonic	*MC1R*	C	T	MC1Rset2R		0.5 µm	TCCAGCAGGAGGATGACG		tttttttttttttACAGCATCGTGACCCTGCCG	0.375 µm
5	rs1805005	16	89985844	Exonic	*MC1R*	G	T	MC1Rset3F	Set3	0.5 µm	GTCCAGCCTCTGCTTCCTG	147bp	tttttttttttttttTGGTGGAGAACG- CGCTGGTG	0.75 µm
6	rs1805006	16	89985918	Exonic	*MC1R*	C	A	MC1Rset3R		0.5 µm	AGCGTGCTGAAGACGACAC		ttttttttttttttttttttCTGCCTGGC- CTTGTCGGA	0.75 µm
7	rs1805007	16	89986117	Exonic	*MC1R*	C	T	MC1Rset4F	Set4	0.4 µm	CAAGAACTTCAACCTCTTTCTCG	106bp	tttttttttttttttttttttttttCTCCATCTTC- TACGCACTG	1 µm
8	rs1805009	16	89986546	Exonic	*MC1R*	G	C	MC1Rset4R		0.4 µm	CACCTCCTTGAGCGTCCTG		ttttttttttttttttttttttttttttttATCTGC- AATGCCATCATC	0.4 µm
9	Y152OCH	16	89986122	Exonic	*MC1R*	C	A						ttttttttttttttttttttttttttttttCATCTT- CTACGCACTGCGCTA	0.6 µm
10	rs2228479	16	89985940	Exonic	*MC1R*	G	A						ttttttttttttttttttttttttttttttttttttC- TGGTGAGCGGGAGCAAC	0.375 µm
11	rs1110400	16	89986130	Exonic	*MC1R*	T	C						ttttttttttttttttttttttttttttttCTTCTA- CGCACTGCGCTACCACAGCA	0.3 µm
12	rs28777	5	33994716	Intronic	*SLC45A2*	A	C	rs28777_F	Set5	0.4 µm	TACTCGTGTGGGAGTTCCAT	150bp	tttttttttttttttttttttttttttttttttttttttCA- TGTGATCCTCACAGCAG	1.2 µm
								rs28777_R		0.4 µm	TCTTTGATGTCCCCTTCGAT			
13	rs16891982	5	33987450	Exonic	*SLC45A2*	G	C	Rs16891982_F	Set6	0.4 µm	TCCAAGTTGTGCTAGACCAGA	128bp	tttttttttttttttttttttttttttttttttttttttttttt- AAACACGGAGTTGATGCA	1 µm
								Rs16891982_R		0.4 µm	CGAAAGAGGAGTCGAGGTTG			
14	rs12821256	12	87852466	Intergenic	*KITLG*	A	G	rs12821256_F	Set7	0.4 µm	ATGCCCAAAGGATAAGGAAT	118bp	ttttttttttttttttttttttttttttttttttttttt- GGAGCCAAGGGCATGTTACTACGGCAC	0.1 µm
								rs12821256_R		0.4 µm	GGAGCCAAGGGCATGTTACT			
15	rs4959270	6	402748	Intergenic	*EXOC2*	C	A	Rs4959270_F	Set8	0.4 µm	TGAGAAATCTACCCCCACGA	140bp	ttttttttttttttttttttttttttttttttttttttttt- GGAACACATCCAAACTATGACACTATG	0.375 µm
								Rs4959270_R		0.4 µm	GTGTTCTTACCCCCTGTGGA			
16	rs12203592	6	341321	Intronic	*IRF4*	C	T	rs12203592_F	Set9	0.4 µm	AGGGCAGCTGATCTCTTCAG	126bp	tttttttttttttttttttttttttttttttttttttttttttt- tTCCACTTTGGTGGGTAAAAGAAGG	0.3 µm
								rs12203592_R		0.4 µm	GCTTCGTCATATGGCTAAACCT			
17	rs1042602	11	88551344	Exonic	*TYR*	G	T	rs1042602 _F	Set10	0.4 µm	CAACACCCATGTTTAACGACA	124bp	ttttttttttttttttttttttttttttttttttttttttttttt- tttttttTCAATGTCTCTCCAGATTTCA	1.25 µm
								rs1042602 _R		0.4 µm	GCTTCATGGGCAAAATCAAT			
18	rs1800407	15	25903913	Exonic	*OCA2*	G	A	rs1800407_F	Set11	0.4 µm	AAGGCTGCCTCTGTTCTACG	124bp	tttttttttttttttttttttttttttttttttttttttttttttt- tttttttttttttttGCATACCGGCTCTCCC	0.1 µm
								rs1800407_R		0.4 µm	CGATGAGACAGAGCATGATGA			
19	rs2402130	14	91870956	Intronic	*SLC24A4*	A	G	rs2402130_F	Set12	0.4 µm	ACCTGTCTCACAGTGCTGCT	150bp	tttttttttttttttttttttttttttttttttttttttttttttttt- ttttttttttttTGAACCATACGGAGCCCGTG	0.75 µm
								rs2402130_R		0.4 µm	TTCACCTCGATGACGATGAT			
20	rs12913832	15	26039213	Intronic	*HERC2*	C	T	rs12913832_F	Set13	0.4 µm	TCAACATCAGGGTAAAA- ATCATGT	150bp	ttttttttttttttttttttttttttttttttttttttttttttttt- tttttttttttttttttTAGCGTGCAGAACTTGACA	1.2 µm
								rs12913832_R		0.4 µm	GGCCCCTGATGATGATAGC			
21	rs2378249	20	32681751	Intronic	*ASIP/PIGU*	T	C	rs2378249_F	Set14	0.4 µm	CGCATAACCCATCCCTCTAA	136bp	tttttttttttttttttttttttttttttttttttttttttttttt- ttttttttttttttttttttCCACACCTCTCCTCAGCCCA	0.18 µm
								rs2378249_R		0.4 µm	CATTGCTTTTCAGCCCACAC			
22	rs12896399	14	91843416	Intergenic	*SLC24A4*	T	G	Rs12896399_F	Set15	0.4 µm	CTGGCGATCCAATTCTTTGT	125bp	ttttttttttttttttttttttttttttttttttttttttttt- ttttttttttttttttttttttTCTTTAGGT- CAGTATATTTTGGG	1.125 µm
								Rs12896399_R		0.4 µm	GACCCTGTGTGAGACCCAGT			
23	rs1393350	11	88650694	Intronic	*TYR*	C	T	Rs1393350_F	Set16	0.4 µm	TTTCTTTATCCCCCTGATGC	124bp	ttttttttttttttttttttttttttttttttttttttttt- ttttttttttttttttttttttttttCATTTGTA- AAAGACCACACAGATTT	1.1 µm
								Rs1393350_R		0.4 µm	GGGAAGGTGAATGATAACACG			
24	rs683	9	12699305	Exonic	*TYRP1*	T	G	rs683_F	Set17	0.4 µm	CACAAAACCACCTGGTTGAA	138bp	ttttttttttttttttttttttttttttttttttttttt- tttttttttttttttttttttttttttGCTTTG- AAAAGTATGCCTAGAACTTTAAT	0.175 µm
								rs683_R		0.4 µm	TGAAAGGGTCTTCCCAGCTT			

Table 2: Information about the 24 DNA variants of the HIrisPlex assay, including PCR and single base extension (SBE) primer sequences and concentrations.

HIrisPlex DNA variants and their use for eye/hair colour prediction

The HIrisPlex assay consists of 24 DNA variants (23 SNPs and 1 INDEL), 6 of these markers, rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs12896399 (*SLC24A4*), rs16891982 (*SLC45A2* (*MATP*)), rs1393350 (*TYR*) and rs12203592 (*IRF4*) are taken from the IrisPlex system which has already been well established [4, 5, 19, 42] and are used for the eye colour prediction part of the HIrisPlex system. The results of these 6 SNPs when their minor allele is input into the HIrisPlex prediction tool are used to predict the eye colour of the individual using the IrisPlex model as previously published [5, 19] with the highest probability of the three categories, brown, blue or intermediate being the predicted eye colour.

The 22 DNA variables used for hair colour prediction are Y152OCH, N29insA, rs1805006, rs11547464, rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, rs1110400 and rs885479 from the *MC1R* gene, rs1042602 (*TYR*), rs4959270 (*EXOC2*), rs28777 (*SLC45A2 (MATP)*), rs683 (*TYRP1*), rs2402130 (*SLC24A4*), rs12821256 (*KITLG*), rs2378249 (*PIGU/ASIP*), rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs16891982 (*SLC45A2* (*MATP*)) and rs12203592 (*IRF4*) based on our previous publication for hair colour prediction [35]. When their minor alleles are input into the HIrisPlex prediction tool, they are used to predict the hair colour of the individual using the HIrisPlex hair prediction model developed in this paper. From the four hair colour categories of blond, brown, red and black, the highest probability value is indicative of the predicted hair colour following guidelines that are published within this paper and described in the next section.

For worldwide hair colour prediction, we assessed the HIrisPlex assay performance on 945 samples from 51 populations of the HGDP-

CEPH set. The MapViewer 7 (Golden Software, Inc., Golden, CO, USA) package was used to plot the predicted hair colour categories and the distribution of SNP genotypes on the world map. A non-metric multidimensional scaling (MDS) plot was produced to illustrate the pairwise F_{ST} distances [43] of the 24 eye and hair colour SNPs between populations, using SPSS 17.0.2 for Windows (SPSS Inc., Chicago, USA). Analysis of molecular variance (AMOVA) (Excoffier 1992) was performed using Arlequin v3.11 [44]. A threshold assessment of prediction probabilities for each hair colour category was also carried out including a combined eye and hair colour prediction probability threshold in the inference of a Non-European individual with Black hair and brown eyes. For the assessment of an age-dependent hair colour change, a Pearson correlation was calculated and the graph plotted using SPSS 17.0.2 for Windows (SPSS Inc., Chicago, USA).

Prediction modelling for hair colour

To develop a hair colour prediction model using samples from several sites with varying levels of hair colour due to their position within Europe, central, western and southern Europe, we took a random subset of 80% of the samples from each site, Poland (n=875), Ireland (n=272) and Greece (n=96). This 80% subset was used to train the model and was based on Multinomial Logistic Regression (MLR), as previously published by Liu et al. [42]. In brief, individuals were categorised according to their hair phenotypes and were split into 4 categories, Blond (n=529), Brown (n=490), Red (n=109) and Black (n=115). For their genotypes, 22 of the 24 HIrisPlex DNA variations (as described above) were used to test for hair colour differentiation and use in the prediction model. By inputting the

minor allele of each DNA variant, including its phenotype and applying MLR, alpha and beta values are generated that form the core of the prediction model. This model then allows the probabilistic prediction of an individuals hair colour category solely based on the input of the 22 variant minor alleles into the HIrisPlex hair colour prediction part of the tool. To assess the effect of the light and dark shades of hair colour that may be contributed from blond and black respectively, a similar approach was used that combined the individuals grouped in the light category (blond, n=529) versus a dark category (black, n=115). Red hair individuals were omitted (n=109) from this analysis as their resulting colour is based upon an *MC1R* cumulative mutation and not on the continuous spectrum of light to dark (i.e. blond to black). Brown hair individuals (n=490) were omitted, as only the extremes of light and dark were required. Therefore using this two-pronged model approach, a predicted hair colour is generated with an approximate indication of the colour being light or dark (i.e. light brown, dark brown) due to the influence of the genotypes commonly associated with the light/dark categories, of blond and black respectively. The further 20% of the combined dataset (total n=308), i.e. from Poland (n=218), Ireland (n=67) and Greece (n=23), was used to assess the accuracy of the prediction model in terms of the final hair colour prediction being correct or incorrect based on colour category, shade and use of the hair colour prediction guide that is described in detail in the Results & Discussion chapter, and an assessment of optimal category thresholds was undertaken. The steps to take when acquiring a prediction based on colour and shade are outlined in a guide provided below.

RESULTS & DISCUSSION

HIrisPlex genotyping assay - design and sensitivity

The HIrisPlex assay was designed with the intention to cope with low template and degraded DNA, a standard concern when genotyping forensic casework samples. Therefore, care was taken to ensure small PCR amplicon sizes of <160 bp for all of the 24 DNA variants considered. During optimisation of the single multiplex assay, a balance of homozygote allele heights and their associated heterozygote allele heights was catered for, to be as consistent as possible when viewing the combined set. With this we aimed to limit the chances of heterozygote dropout at the lower concentration levels. For the INDEL variant N29insA (first peak in the assay, Figure 1) however, the peak height is lower, on average by a factor of 2 depending on the sample DNA input, relative to the 23 SNPs in the multiplex. This is due to difficulties within the design that is known to occur with INDELs. Nevertheless, this does not affect the assay until the very low DNA input levels (<63 pg) for which sensitivity was assessed. Notably, N29insA is extremely rare in the prediction of red hair individuals alone; only 4 out of a total 137 red hair phenotype individuals had this mutation in our dataset. Hence, in most of the cases, this technical issue is not likely to affect the practical use of the HIrisPlex assay. If, however, allelic drop-out for N29insA is indeed observed in a case, N29insA shall be genotyped using the more sensitive singleplex assay to take full advantage of the red hair colour prediction available with the marker set considered here. Our population studies revealed that DNA inputs of >500pg usually yield a balanced profile with high relative fluorescence units (rfu) levels, especially for homozygote SNP alleles.

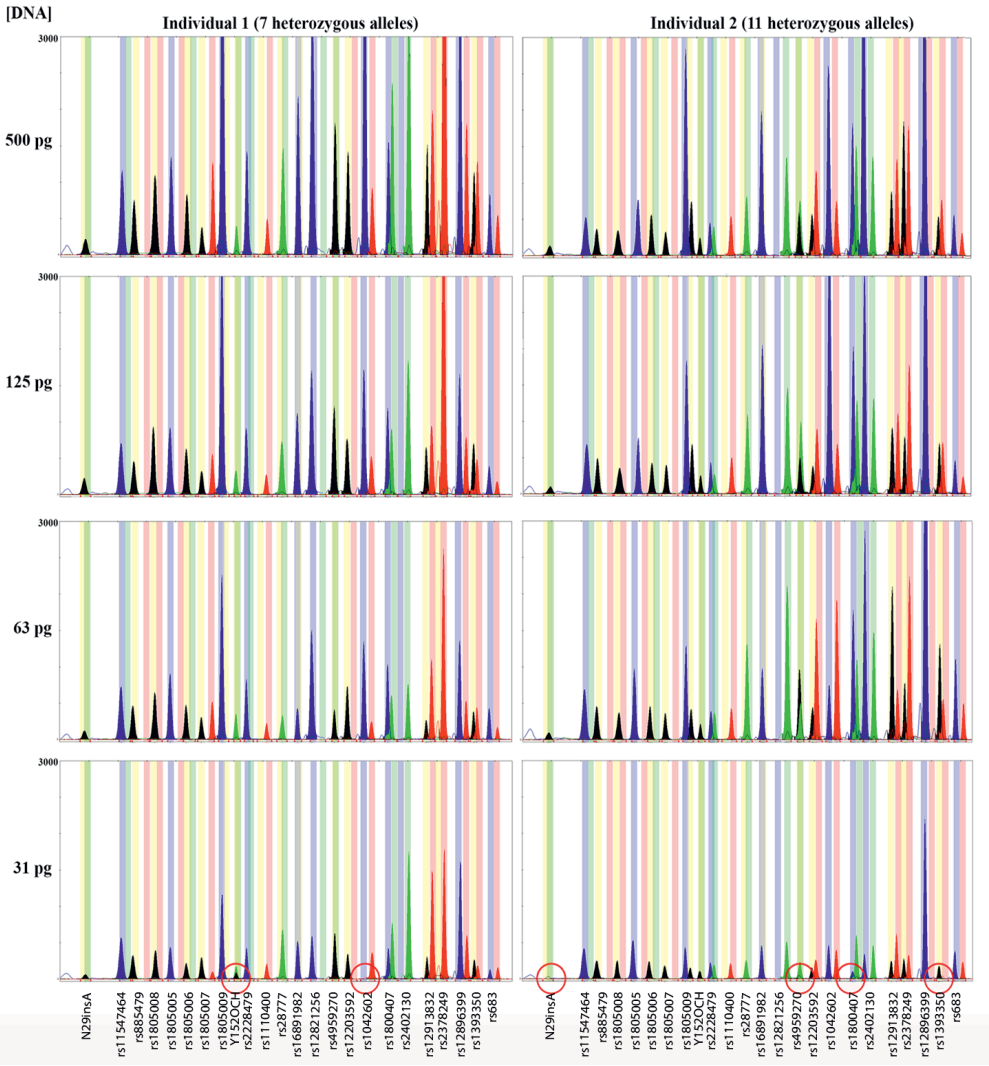

Figure 1: An assessment of the HIrisPlex assay's sensitivity on two individuals ascertained to have high numbers of heterozygote alleles (7 and 11, respectively) for quantified DNA input at 500pg, 125pg, 63pg and 31pg. Full profiles were observed down to 63pg DNA input with drop out occurring at 31pg DNA input for insertion 1 (N29insA), SNP 15 (rs4959270), SNP 17 (rs1042602), SNP 18 (rs1800407), and SNP 23 (rs1393350) including a C allele drop in at SNP 9 (Y152OCH).

For a first investigation of the sensitivity threshold of the HIrisPlex assay, two individuals were genotyped in a duplicate dilution series of DNA input at 500pg, 250pg, 125pg, 63pg, and 31pg, established after DNA quantification at 500pg using Quantifiler Human DNA Quantification kit (Applied Biosystems). These individuals were chosen for maximizing the heterozygous state of the 24 DNA variants, which is important, as signals from heterozygote alleles are not as strong as homozygote alleles for the same marker. From Figure 1 it is evident that at 500 pg and lower, peak height imbalance occurs and this should be taken into account when assessing genotype calls at these lower DNA levels; however, genotype accuracy is not affected until very low DNA input levels. Peak imbalance can sometimes be confused with the possibility of a DNA mixture from different individuals; but it is important to note here that in most circumstances HIrisPlex will be used after an STR profile has been generated from crime scene material (and found not to be informative), therefore the presence of a DNA mixture should be evident from the STR profile. The sensitivity of the 24 HIrisPlex assay is high, with full profiles observed at DNA input levels down to and including 63pg, while allele drop out occurs at the lowest examined level of 31pg DNA input for some HIrisPlex DNA variants (Figure 1). In particular, dropout was observed in 5 instances for this set of profiles, at N29insA, rs1042602, rs4959270, rs1800407 and rs1393350. One drop-in occurred at 31 pg starting DNA of a C allele at Y152OCH.

Overall, the HIrisPlex assays sensitivity, according to the preliminary assessment done here, is comparable to some other complex SNaPshot™ assays such as an 18-plex designed by Freire-Aradas et al. [45] for human individual identification from highly degraded DNA using

autosomal SNPs. For that assay, full profiles down to 78 pg/µl DNA input were observed with partial profiles down to 31 pg DNA input, as similar for the HIrisPlex assay. These minimal input levels are lower than those reported for other autosomal SNP assays such as the two multiplex assays together covering 44 SNPs for individual identification by Lou et al [46] where a DNA input of at least 125 pg is needed to receive a full profile. Notably, our previously developed IrisPlex assay that includes 6 eye colour predictive SNPs gave full profiles down to a level of about 31 pg input DNA [5], which is slightly more sensitive than the HIrisPlex assay presented here. This is at least partly explained by the 4 times larger number of DNA variants included in the HIrisPlex assay relative to the previously developed IrisPlex assay. For practical applications this may mean that if allelic dropout due to low quality/quantity input DNA is indicated by complete locus drop-out at any of the 6 HIrisPlex SNPs for eye colour, the more sensitive IrisPlex assay may be applied subsequently and may provide a full 6-SNP profile for eye colour prediction on critical DNA samples.

HIrisPlex model-based hair colour prediction

MC1R polymorphisms are largely recessive when considered individually, but also interact with each other through a genetic mechanism known as ''compound heterozygosity'' [47-49]. In our previous population-based hair colour prediction study [35], the *MC1R* variants Y152OCH, N29insA, rs1805006, rs11547464, rs1805007, rs1805008, rs1805009, rs1805005, rs2228479, rs1110400 and rs885479 were all collapsed into two markers, *MC1R*-R (R/R, R/wt, wt/wt) and *MC1R*-r (r/r, r/wt, wt/wt), depending on the penetrance of the mutant alleles. Thus, the total 22 hair colour markers

were considered as 13 markers in our previous prediction analysis, including, *MC1R*_R, *MC1R*_r, rs1042602 (*TYR*), rs4959270 (*EXOC2*), rs28777 (*SLC45A2 (MATP)*), rs683 (*TYRP1*), rs2402130 (*SLC24A4*), rs12821256 (*KITLG*), rs2378249 (*PIGU/ASIP*), rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs16891982 (*SLC45A2* (*MATP*)) and rs12203592 (*IRF4*). In the current study, we had two main reasons for the development of a new hair colour prediction model utilising a 22 DNA variant set. First, we were able to produce a larger dataset that provides a broader representation of Europe and its highly variable hair colour regions. Notably, we not only increased the sample size relative to our previous study [35] by 3-fold, but in addition to considering more Eastern Europeans from Poland (as also used before) we also added individuals from Western Europe i.e., Ireland and from Southern Europe i.e., Greece. These three countries display very different hair colour phenotype frequencies (Table 1), which would also impact on the modelling. The use of samples from three European regions and countries provides an increase in overall sample size and also a better representation of the hair colour phenotype variation across Europe, but this also increases the different genotype combinations observable. Second, some of the *MC1R* variants also contribute to hair colours other than red [35] (as seen in Table 3). As many individuals from Ireland display a higher frequency of *MC1R* mutations, up to 75% noted in a previous study with 30% of these being double mutations [50] and 78% in our own set contain at least one of the *MC1R* mutations without displaying the red hair phenotype, other Europeans from other regions to which our hair colour prediction tool may be applied in the future may also reflect this. Therefore, a new hair colour prediction model was developed to examine the input of each single DNA variation for hair colour categorical

prediction, including the individual impact of all *MC1R* variants separately.

Figure 2 shows a hypothesised tree model illustrating how each of the 22 DNA variants contributes towards a categorical hair colour prediction as inferred from our current data. This scenario represents the extreme of a 2 minor allele input for each single DNA variant and the largest single hair colour category effect that is seen on the models prediction, based on that input. However, it is important to note here (and as further outlined below) that it is the combination of all 22 DNA variants together in a single model that finally allows the prediction of hair colours as we suggest here with this study.

Table 3 provides a measure of the strength of each DNA variants contribution towards each hair colour category prediction using beta values including p-values obtained from the MLR model. The analysis is based on the combined 80% model-building subset of 1243 Polish, Irish and Greek individuals assigned into a red *versus* non-red colour category which then displays each DNA variant's contribution towards red hair colour within the model. For the other categories (i.e. blond *versus* non-blond, brown *versus* non-brown and black *versus* non-black), we used a total set of 1134 individuals representing the 80% model-building subset but now omitting the red hair individuals from the analyses due to their rare DNA variants and the fact that red hair is not a continuous colour but more a combined *MC1R* mutation effect on colour change [47, 51]. As the probability values shown suggest, the results for hair colour variation from blond via brown to black (without red) are consistent with our previous findings [35] in several DNA variants, i.e. rs12913832 (*HERC2*) and rs12203592 (*IRF4*) with high statistical support (P 10^{-6} to 10^{-16}) in the present enlarged dataset considering Poland, Ireland and Greece.

Number	DNA variant	Gene	Blond(beta)	Blond(p)	Brown(beta)	Brown(p)	Black(beta)	Black(p)	Red(beta)	Red(p)
1	N29insA	MC1R	–	–	–	–	–	–	-21.9731	0.994026
2	rs11547464	MC1R	–0.947299	0.081175	–0.4007191	0.441688	–16.782634	0.995907	–2.8866	4.42E–08
3	rs885479	MC1R	0.272536	3.36E–01	0.1938828	0.460717	2.29E–01	0.575679	0.315529	0.707292
4	rs1805008	MC1R	–0.57034	0.003874	–0.3058868	0.097798	–5.66E–01	0.084668	–3.02472	2.20E–16
5	rs1805005	MC1R	0.20689	2.28E–01	0.2382036	0.128146	–1.57E–01	0.539306	–0.86742	0.025064
6	rs1805006	MC1R	1.718508	0.045418	2.1268136	0.009857	–1.70E+01	0.996356	–2.43626	0.001714
7	rs1805007	MC1R	–0.53542	0.030279	–0.1503278	0.508278	–1.32E+00	0.009567	–3.59956	2.20E–16
8	rs1805009	MC1R	0.550547	5.60E–01	0.5309897	0.49513	–4.70E–01	0.693758	–4.25774	4.14E–08
9	Y152OCH	MC1R	–	–	–	–	–	–	–19.3501	0.992969
10	rs2228479	MC1R	–0.025643	8.83E–01	–0.1128742	0.483857	1.98E–01	0.413966	–0.61967	0.110936
11	rs1110400	MC1R	–0.366071	0.338334	–0.5920858	0.123046	6.63E–01	0.21252	–1.67775	0.009302
12	rs28777	SLC45A2	0.566568	0.414238	0.3138274	0.561428	4.85E–01	0.468883	–0.41607	0.743869
13	rs16891982	SLC45A2	0.863795	0.194837	0.2562763	0.618846	6.29E–01	0.326034	0.891013	0.522114
14	rs12821256	KITLG	–0.434962	0.020898	–0.1743193	0.32142	–6.87E–01	0.056556	0.406751	0.312582
15	rs4959270	EXOC2	–0.251437	0.019073	–0.1555227	0.120958	–2.71E–01	0.104087	–0.34639	0.107774
16	rs12203592	IRF4	1.741377	2.20E–16	1.0810914	2.22E–16	8.80E–01	2.35E 06	0.071132	0.773323
17	rs1042602	TYR	0.125113	0.24551	0.141479	0.155781	–4.52E–02	0.779493	–0.3842	0.071464
18	rs1800407	OCA2	–0.204189	0.331948	–0.0048133	0.97935	–3.53E–01	0.202517	0.223931	0.580501
19	rs2402130	SLC24A4	0.354085	0.00797	0.2752735	0.023746	4.36E–02	0.820086	–0.08861	0.724429
20	rs12913832	HERC2	1.372353	2.20E–16	0.6797949	6.83E–10	1.19E+00	6.65E 13	0.754729	0.004319
21	rs2378249	PIGU/ASIP	0.088319	0.526489	0.1828612	0.154928	–1.64E–01	0.449722	–0.72184	0.002302
22	rs683	TYRP1	0.197865	0.066913	0.168184	0.08995	1.58E–03	0.992081	0.129235	0.540918

Table 3: Assessment of the contribution of each HIrisPlex DNA variant for hair colour prediction within the model in terms of betas and probability (p) values. The values generated reflect a binary category assessment of colour prediction, i.e. blond versus non-blond, brown versus non-brown etc. The lowest (and thus most statistically significant) p values for each category are highlighted for the respectively associated DNA variants.

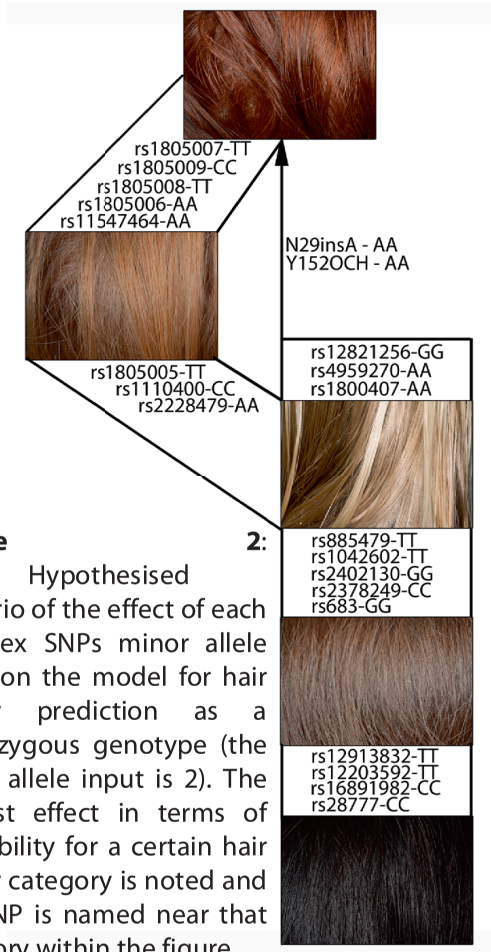

Figure 2: Hypothesised scenario of the effect of each HIrisPlex SNPs minor allele input on the model for hair colour prediction as a homozygous genotype (the minor allele input is 2). The highest effect in terms of probability for a certain hair colour category is noted and the SNP is named near that category within the figure.

Although less powerful, additional DNA variants also show significant evidence (P<0.05) for some hair colours, such as rs2402130 (*SLC24A4*), rs12821256 (*KITLG*), rs4959270 (*EXOC2*), rs1805006 (*MC1R*), rs1805007 (*MC1R*), rs1805008 (*MC1R*) for blond, rs1805006 (*MC1R*) and rs2402130 (*SLC24A4*) for brown, and rs1805007 (*MC1R*) for black. Red hair colour prediction is observed with highest probability values (P 10^{-8} to 10^{-16}) for several of the individually considered *MC1R* variants as expected i.e., rs1805008, rs1805007, rs1805009 and rs11547464, and with somewhat less statistical strength (P<0.05) for other *MC1R* variants i.e., rs1805005, rs1805006 and rs1110400. However, due to the very low frequency in our set of individuals of the generally rare *MC1R* variant allele at N29insA (INDEL) and Y152OCH, their contribution towards red hair probabilities are particularly high (Table 3; red hair beta values of -22 and -19.4 respectively) i.e., the presence of an A allele at N29insA or Y152OCH produces red hair prediction probabilities of 1. This effect is not mirrored in the other *MC1R* variants investigated and reflects the presence of these very rare alleles (heterozygote and homozygote state) within all individuals displaying a red hair phenotype in our model training set at a very low frequency (n=6). Although this does not affect the final prediction of red hair, it is important to note the abnormally high probability values for red when these rare variants are present. Notably, some DNA variants outside the *MC1R* gene also show significant red hair colour probabilities (P<0.05) i.e. rs12913832 (*HERC2*), and rs2378249 (*PIGU/ASIP*).

Figure 3 provides the results of HIrisPlex prediction for a subset of 44 Irish individuals where high-resolution non-dyed hair colour imagery was available to illustrate the model's performance. The individuals

natural hair colour images were ordered according to their predicted hair colour category probability values achieved via HIrisPlex analysis while the actual hair colour phenotypes were not considered in the ordering. From left to right, top to bottom, the images are ordered from the highest to lowest HIrisPlex prediction probabilities for black hair and then the lowest to highest prediction probabilities for brown, red and blond hair respectively. As evident, there is a high correlation with the predicted hair colour category from HIrisPlex and the hair colour phenotype observed from visual inspection of these images.

Table 4 shows the accuracy of hair colour prediction in the 20% model-testing subset of the Polish, Irish, and Greek individuals (N=308). It is important to emphasise here that these individuals were not used for model building. The highest probability category approach (as opposed to the prediction-guide approach explained in the next paragraph) considers the colour category with the highest predicted probability as the final predicted colour and does not take other categories into account for the final prediction. Using this approach, we tested various probability thresholds, from no threshold, to $p>0.7$, which we previously recommended for eye colour prediction using the IrisPlex system [4, 19]. As seen in Table 4, using the $p>0.7$ (B) threshold increases the percentage of correct calls relative to the value obtained without using any threshold (A) for some hair colours such as red hair by ~10% (i.e., from 89.5 without threshold to 100% with threshold), and for blond hair by ~6.5% (i.e., from 57.2 to 63.6%), whereas no difference was seen for brown hair at 75%, and for black we saw a decrease by ~8.5% (i.e. from 28.6 to 20%). The low prediction accuracy obtained with this approach for black hair may reflect the difficulty of defining the true black hair colour phenotype relative to

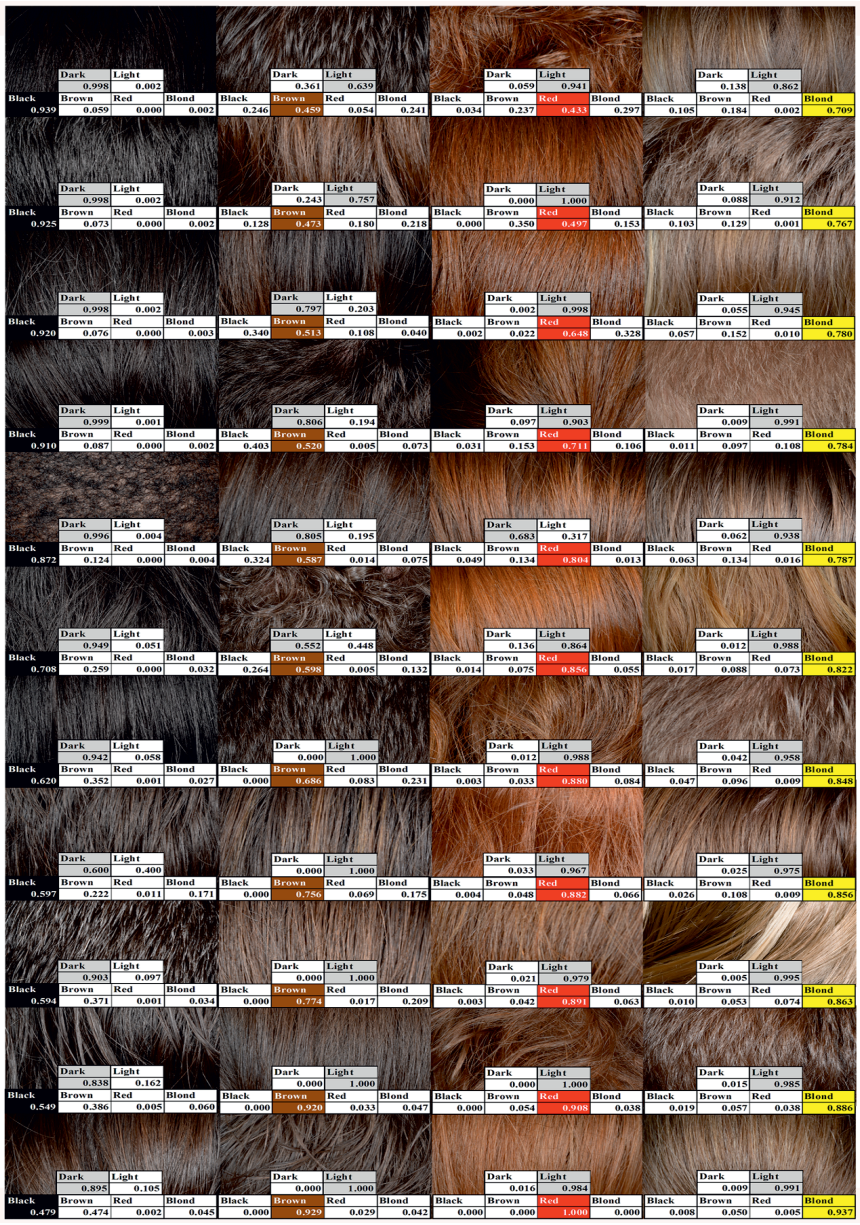

Figure 3: Illustrative example of 44 individuals on the model performance of the HIrisPlex system for hair colour prediction. The 44 individual set was taken from the Irish collection for which hair imagery was noted as neither grey nor dyed. These individuals are only a visual example of how the model performs. Probability values are given for all four hair-colour categories (black, brown, red and blond) with the highest probability value and the category for which a colour is called highlighted. Dark and light probability values are also indicated which show the amount of black and blond contribution and effect towards the final colour prediction using the guide in Figure 4. The individual hair figures are ordered from left to right, top to bottom, starting with the highest probabilities for black to the lowest in column one, for the remaining columns the order is the lowest to the highest probabilities for brown, red and blond, respectively.

Highest probability category approach	Observed categories				
No threshold p value	Red	Blond	Brown	Black	Total prediction(n)
Red predicted	17(89.5%)	1(5.3%)	1(5.3%)	0(0%)	19(100%)
Blond predicted	8(3.7%)	123(57.2%)	68(31.6%)	16(7.4%)	215(100%)
Brown predicted	2(6.3%)	5(15.6%)	24(75%)	1(3.1%)	32(100%)
Black predicted	1(2.4%)	2(4.8%)	27(64.3%)	12(28.6%)	42(100%)
Total phenotype(n)	28	131	120	29	308 Total

Highest probability category approach	Observed categories				
>0.7p threshold	Red	Blond	Brown	Black	Total prediction(n)
Red predicted	8(100%)	0(0%)	0(0%)	0(0%)	8(100%)
Blond predicted	2(1.8%)	70(63.6%)	33(30%)	5(4.6%)	110(100%)
Brown predicted	0(0%)	0(0%)	3(75%)	1(25%)	4(100%)
Black predicted	0(0%)	0(0%)	4(80%)	1(20%)	5(100%)
Total phenotype(n)	10	70	40	7	127 Total
Undefined	**18(64.3%)**	**61(46.6%)**	**80(66.7%)**	**22(75.9%)**	**181 (58.8%)**

	Observed categories					
Prediction guide approach	Red	Blond	d-blond/l-brown	D-Brown	Black	Total prediction (n)
Red predicted	24(80%)		6(20%)	0(0%)	0(0%)	30(100%)
Blond/d blond predicted	2(1.7%)	32(26.4%)	52(43%)	30(24.8%)	5(4.1%)	121(100%)
L-brown/d-brown predicted	2(1.3%)	9(6%)	59(39.6%)	58(38.9%)	21(14.1%)	149(100%)
Black/d-brown predicted	0		1(12.5%)	4(50%)	3(37.5%)	8(100%)
Total phenotype (n)	28	41	118	92		29 308 Total

Table 4: HIrisPlex hair colour prediction accuracies obtained from a 308 separate model testing set of individuals from Poland, Ireland and Greece (individuals were not considered for prediction model building for which a different set of 1243 individuals was used) using two approaches: the highest probability category approach (with and without thresholds) and the prediction guide approach (see Figure 4 for the prediction guide)

the dark brown phenotype within this European dataset, where black hair is rare. Notably, the low correct call rate of 28.6% for black (without using a threshold) is mainly caused by 30 individuals with non-black self-reported phenotypes that were predicted as black by the HIrisPlex model. Of these, almost all (i.e. 90%) had the brown-dark brown phenotype. We could speculate that at least some of them may have been self-categorised as black if black hair colour would be more frequent in the sampled populations and therefore easier to differentiate from dark brown in the phenotyping procedure. Although red hair is also rare in the European population (albeit in our Polish dataset it was enriched for) this problem is less expected for red hair as red is usually well differentiable from other hair colours, perhaps with the exception of the blond-red individuals. The prediction accuracy for blond hair, being lower than those

for red and brown hair colour with and without threshold, is partly due to another phenomenon that will be discussed in detail in chapter 3.3; age-dependent hair colour changes. As brown hair is the intermediary stage between blond and black, no prediction threshold for this category is required as can be seen in Table 4. Even at the 75% correct call rate, the incorrect 5/8 defined themselves as being dark blond. Since we know an overlap exists between light-brown and dark-blond in people's perception and definition of colour, it is best to consider dark blond the same colour as light brown. Therefore, brown hair colour may also be seen at black and blond category predictions < 0.7p depending on their light and dark shade predictions and this is where the use of the prediction guide (see next paragraph) is more informative. For the red hair category, as its occurrence is independent of the continuous spectrum of dark to light (black to blond), and mutations in the *MC1R* gene produce a prediction within the category of red hair, all (with >0.7p threshold) or nearly all (89.5% without threshold) individuals for which the red hair category was the highest prediction probability were correctly predicted as seen in Table 4. Notably, the two individuals that were incorrectly predicted red without using a threshold defined themselves as blond and brown, respectively; upon inspection of a hair image of the latter individual that was available to us, it did in fact display light red hints of colour. This reflects another example of how the phenotyping procedure, particularly self-reported hair colour grading as done in our Irish and Greek datasets, influences DNA prediction accuracy. However, it is important to point out here that for 11 (39%) individuals that had defined themselves as having red hair, the red hair probability was not the highest, relative to probabilities for non-red hair colour, and these

individuals were therefore missed out with HIrisPlex using this highest-probability approach. Furthermore, for 8 (6%) of the phenotypic blond, 96 (80%) of the phenotypic brown, and 17 (59%) of the phenotypic black hair individuals the highest predicted hair colour category did not correspond to the phenotypic hair colour category and hence these individuals were missed using this highest-probability approach. This illustrates the limitation with the highest-probability approach that we aimed to overcome by developing and applying a prediction-guide approach as discussed next.

To take full advantage of the genotype-phenotype relationship for hair colour and the 22 hair-colour predictive DNA variants included in the HIrisPlex system we developed a hair colour prediction guide considering categorical hair colour probabilities in combination with light/dark hair colour shade probabilities as obtained from the HIrisPlex genotype data (Figure 4, see also chapter 3.5 for additional practical recommendations). The reason for considering light/dark shade prediction in addition to categorical hair colour prediction in the final approach is that the 22 DNA variants not only impact on the main hair colour categories, but also on more detailed hair colour information, which is difficult to measure; hence, we express in light/dark prediction probabilities. For this, we took the individuals from the black category, now termed dark, and the individuals from the blond category, now termed light, and designed an additional prediction model for light and dark colour shade. Therefore, the HIrisPlex genotype input finally provides the core prediction colour category with an added level or shade i.e., light or dark. This part of the prediction should be useful as additional information to the initial prediction category e.g. to differentiate light blond from dark blond (light

brown), or light brown from dark brown/black.

It becomes particularly beneficial in the lower hair colour category prediction probability levels (i.e. category prediction < 0.7 p for non-red) as the categories are closer together and may be more difficult to accurately predict one category over another due to genotype combinations. A >0.9 threshold is used for light versus dark shade prediction. As seen in Table 4(C), using the prediction guide approach the correct call percentages were for all hair colours considerably higher than using the highest probability category approach, except for red hair. In fact, using the prediction guide approach we obtained on average 69.5% correct calls for blond, 78.5% for brown and 87.5% for black. Particularly black hair prediction was strongly improved by using the prediction guide approach with an increase of almost 60% on average relative to the highest probability category approach without a threshold. For an explanation of why blond is the least accurately predictable hair colour with currently available DNA markers, also after applying the prediction guide, see chapter 3.3. Although we saw an apparent decrease of accurate prediction for red hair with the prediction guide approach (80% versus 89.5% with highest-probability approach without threshold), this can be explained by the total number of red predictions made by the models and if they were correct or not. In particular, for the highest probability approach the model was incorrect at predicting red only 2 times but missed out on 11 actual reds from our dataset. The prediction guide approach, although was inaccurate for red hair prediction for 6 individuals, it managed to predict 24 out of the 28 actual red hair phenotypes from our test set.

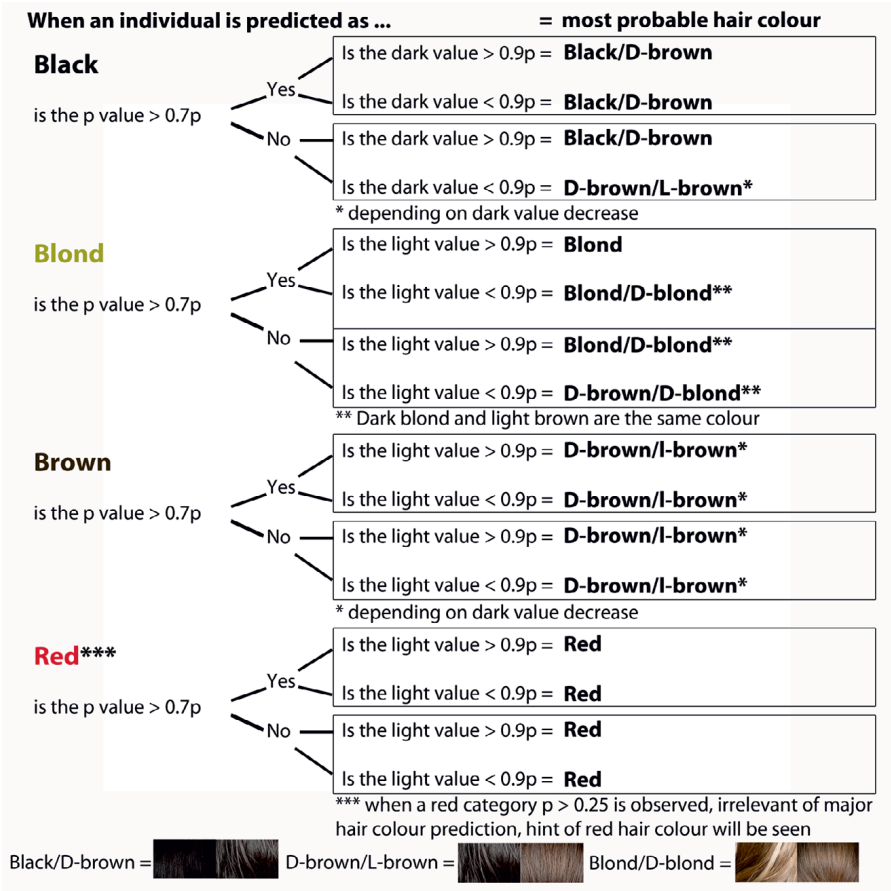

Figure 4: HIrisPlex prediction guide on how to interpret individual hair colour and hair shade probabilities as derived from the HIrisPlex prediction tool available via Supplementary Table 2. D-brown stands for dark brown and l-brown stands for light brown.

In summary, the number of individuals in our 308 model-test set that were missed by HIrisPlex hair colour prediction using the prediction guide approach were 4 (14%) of the phenotypic red, 8 (19.5%) of the phenotypic blond, 7 (6%) of the phenotypic d-blond/l-brown, 28 (31%) of the phenotypic d-brown and 26 (90%) of the phenotypic black, with an overall hair colour prediction accuracy of 76%. All are considerably less than what

was missed when applying the highest probability category approach, apart from black hair where we believe phenotyping
inaccuracy / perception of colour plays a role as discussed above already, as 21 of those individuals were predicted as having d-brown hair and may have in fact displayed d-brown hair that was perceived as black within Europe. We therefore recommend using the prediction guide approach for properly interpreting HIrisPlex genotype data and the probability values derived from our prediction tool to infer the most likely hair colour phenotype in future practical applications.

There are several important differences between eye and hair colour, both on the phenotypic as we ll as the genotypic levels, that may play a role in why some eye colours (i.e., blue and brown) appear to be currently predictable from DNA with higher accuracy than some hair colours (i.e. all non-red hair colours). Rs12913832 from the *HERC2* gene plays a major role in the functional aspects of iris pigmentation [10, 52] and its proposed model of action reflects a type of on/off switch from the absence of the T allele (and the homozygous presence of the C-allele) resulting in blue eye colour, to the presence of one or two T allele(s) reflecting brown eye colour [10]. Indeed, it has been shown recently [52] via a series of functional genetic experiments that the rs12913832 T-allele leads to binding of several transcription factors and a chromatin loop with the promoter of the neighbouring pigmentation gene *OCA2* leading to elevated *OCA2* expression and dark pigmentation. In contrast, when the rs12913832 C-allele is present, transcription factor binding, loop formation and *OCA2* expression are all reduced leading to light pigmentation. Because of its strong functional involvement, *HERC2* rs12913832 shows

the strongest predictive power on categorical eye colour with an AUC of 0.877 for blue and 0.899 for brown alone for this SNP [42], and it also shows a strong impact on quantitative eye colour variance explaining on average 46% of the H and S spectrum [53]. When comparing this to its impact on hair colour, the percentage of residual variation from black to blond explained by *HERC2* rs12913832 was 10.7% in a previous study [8]. However, the effect of rs12913832 is considerably less on hair colour than it is on eye colour for reasons yet to be unveiled, and there are no other high impact hair colour SNP that take its place. For instance, in our full dataset using 1551 individuals, the correlation of rs12913832 with eye colour is nearly twice as high (Pearson correlation $r^2 = 0.46$, P = 2.2e-16) as its correlation with hair colour (Pearson correlation $r^2 = 0.24$, P = 2.2e-16). Furthermore, the colour distribution of European hair appears much wider than that of European eyes, requiring the combination of several similar gene effects [54]. Thus, categorical hair colour prediction is expected to be more error-prone especially when involving factors such as shade and intensity etc. at least with the DNA markers known thus far. Additional effects such as environmental contributions particularly life time that are much stronger on certain hair colours than they are on all eye colours also influence hair colour prediction accuracy more so than eye colour prediction accuracy and will be discussed in the following chapter (see 3.3).

Age-dependent hair colour changes and consequences for hair colour prediction

Age-dependent changes in hair colour are evident from anecdotal knowledge. The most often observed age-dependent hair colour changes

occurs from light blond during childhood towards dark blond / light brown as an adult, but can also occur from light brown to dark brown/almost black. Suggestions of hormonal changes during adolescence have been advocated as a possible explanation [55], but the molecular basis are yet to be unveiled. In order to study the effect of age-dependent hair colour change on hair colour prediction from child to adulthood we recorded via questionnaires in the Irish sample set hair colour during childhood and adulthood separately, including the approximate age of the hair colour change. Of the 339 Irish individuals, 157 contained current images in which the hair was not dyed and not grey, and from these the 8 individuals that were classified as blond in adulthood were 100% correctly predicted by the HIrisPlex system following the prediction guide approach. However, for 14 individuals with light brown to black phenotypes the HIrisPlex model had faltered and gave a high blond prediction probability (>0.7 p) with high light shade probabilities (>0.9 p). On further examination of these incorrectly predicted individuals, 8 (57%) of them noted that a change in hair colour regarding a darkening from blond to brown had occurred in their younger lives at ages ranging from 9 to 12 years. Furthermore, we found a high and statistically significant correlation (Pearson correlation r^2=0.81, P <0.01) between the increase in brown (darkening of hair) and the increase in age since the hair colour change occurred for those Irish individuals for whom such data were available to us (Figure 5), which substantiates that the hair colour change observed is age dependent in these individuals.

From this data we can see that our current HIrisPlex system works to a high degree of accuracy for hair colour prediction, but there may be processes that alter the hair colour over an individual's lifetime (possibly

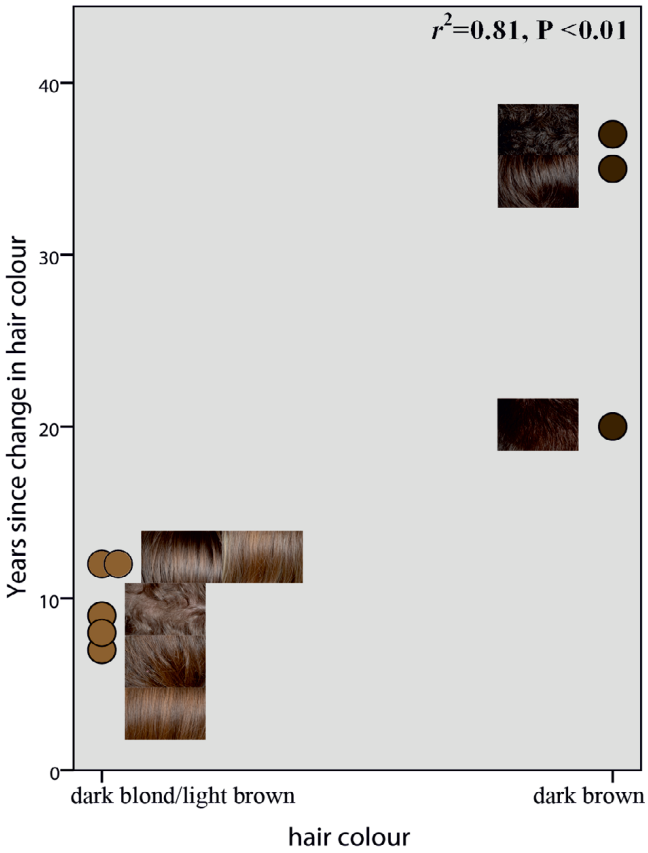

Figure 5: A Pearson correlation plot illustrating the age dependent hair colour change in years versus the darkening of hair colour. Individuals are from the Irish set and display a dark blond/light brown to dark brown phenotype depending on the number of years since the change occurred, while all were noted as having light blond hair as a child.

molecular processes) without changing the HIrisPlex predicted hair colour of the individual. For instance, an adult that had blond hair as a young child, but now displays light-dark brown/black hair colour is likely to display blond HIrisPlex genotypes and therefore a blond hair colour prediction will be obtained. This is due to the fact that the hair colour SNPs included in the HIrisPlex system, as well as any additional hair colour associated DNA variant today, were identified in studies dealing with adults, and not in studies that particularly searched for bio-markers informative for the age-dependent hair colour change, which is still yet to be carried out. It is important to note therefore that the HIrisPlex model cannot decipher between these change-affected individuals and blonds who remain blonds from childhood to adulthood and thus a HIrisPlex

prediction of blond hair may be inaccurate to a certain degree (30% (Supplementary Table 3) in our dataset). This limitation in DNA-based hair colour prediction will remain as long as bio-markers informative for indicating age-dependent hair colour changes are not identified. Furthermore, this age-dependent study was conducted using images solely taken from a small Irish set (childhood hair colour was not available for the Polish and the Greek set); it is worth mentioning that this may reflect a trend in other countries within Europe, however we do not have this information as of present. Therefore more samples and increased accuracy testing of the HIrisPlex system on a broader collection around Europe would be advantageous to get a better measure of this phenomenon. Furthermore, finding the processes/genes responsible and developing respective bio-markers that may increase hair colour prediction accuracy.

A different aspect of age-dependent hair colour change is the loss of hair colour when turning grey and white at a more or less advanced age, which likely represents a different mechanism of action [56] than changing from one hair colour to another. We examined the Irish population of 339 individuals for which we had questionnaire information on the age at which grey or white hairs had started to grow. As shown in Supplementary Figure 1, after the age of 30 there are more individuals starting to produce grey or white hairs relative to those who do not, confirming anecdotal knowledge. However, we have no data on how long it will take for those individuals who started to have grey hairs to turn grey to a substantially obvious phenotypic degree. For practical considerations, knowing the natural hair colour for an individual (during its youth <40 on average) that now at more of an advanced age displays an obvious grey or

white hair phenotype will not be directly useful in an investigative search, but this information can still be useful albeit less strongly, when asked for natural hair colour prior to greying in these questionable individuals during a police inquiry. For differentiating whether a crime scene sample donor still had his/her natural hair colour, or perhaps turned grey or white already, a molecular age estimation performed on crime scene samples such as blood would be useful in combination with the HIrisPlex application. Previously, our group developed a DNA test for chronological age, which allows age-group estimation on an accurate level [57]. Obviously, any dyed hair colour, as long as it produces a hair colour different from the natural hair colour category, would not be identifiable with HIrisplex or any other DNA-based hair colour prediction tool. However, in general it is believed that many people who dye their hair as a result of hair greying, and with the intention of hiding the fact that their hair has greyed, try to achieve their natural hair colour category via dyeing, especially in the case of men, to avoid stigmatisms associated with hair colouring. In such cases HIrisPlex hair colour prediction can still be useful even though the hair is dyed.

HIrisPlex analysis on a worldwide scale

Due to the fact that the HIrisPlex hair prediction model was created using individuals solely from Europe, as it should be for a European trait, to verify its use outside of Europe we performed HIrisPlex analysis on worldwide DNA samples from the H952 subset of the HGDP-CEPH panel that represents 952 individuals from 51 populations [36, 37]. Due to lack of DNA in some samples, a final number of 945 worldwide samples were used. Figure 6 displays the prediction of the four hair-colour categories

blond, brown, red and black on a worldwide scale. This figure does not use any threshold parameters and therefore it is worthy to note that the prediction levels of blond hair in Europe (especially with probability values <0.7 p) may reflect more of a brown hair colour prediction upon inspection of the probability values and the prediction chart that should be used in Figure 4. Although the actual hair colour of the HGDP-CEPH individuals is not known, we conform to general knowledge that individuals distant from Europe and its neighbouring regions (i.e., Middle East and parts of West Asia) display a black hair colour phenotype as illustrated by proposed figures of hair colour distribution [27], (with a image depiction found at http://cogweb.ucla.edu/ep/Frost_06.html.). As seen from Figure 6, for every individual who originates from regions that are distant from Europe and neighbouring regions, namely East Asia, Oceania, Sub-Saharan Africa and the Americas where only black hair is assumed to be present, HIrisPlex indeed predicts black hair as the only hair colour with no exception. Only in Europe, Russia, Israel and parts of Pakistan, the region covered by HGDP-CEPH samples where hair colour variation is assumed to be present, HIrisPlex predicts individuals with red, blond, brown as well as black hair colour.

This mirrors our earlier findings using the IrisPlex system for worldwide eye colour prediction, where only brown eye colour was predicted in East Asia, Oceania, Sub-Saharan Africa and the Americas (with a single exception of an individual below the 0.7p threshold level but still displayed a brown eye colour prediction); i.e., the worldwide regions where only brown eyes are assumed to exist. Also in Europe, Russia, Israel and parts of Pakistan where there is assumed eye colour variation, IrisPlex

indeed predicted blue, intermediate and brown eye colour [19]. These results suggest that HIrisPlex hair and eye colour prediction is reliable on the worldwide scale and highlights that HIrisPlex hair and eye colour prediction can be applied independently from bio-geographic ancestry knowledge and without the need for extra DNA ancestry testing in practical applications such as forensics.

Furthermore, we examined the effect of the 24 DNA variants included in the HIrisPlex system on their potent ability to infer biogeographic ancestry. It had been advocated before that SNPs from pigmentation genes are useful for genetic ancestry detection [58]. Previously we had shown that the 6 SNPs from the IrisPlex system were able to separate Europeans from Non-Europeans to a certain degree on the population (but not necessarily on the individual) level [4]. Figure 7 shows a two-dimensional plot from a non-metric multidimensional scaling (MDS) analysis of pairwise F_{ST} values estimated between pairs of all the 51 HGDP-CEPH populations using the 24 DNA variants of the HIrisPlex system (*S*-stress value 0.04030). As evident, the 1st dimension separates the European populations (except Sardinians and Adygei) from all non-European populations with all Middle-Eastern populations and the Kalash from Pakistan. Hence almost all groups with predicted hair colour variation are clustered closer to the European groups, whereas the colour variation are clustered closer to the European groups, whereas the East Asian groups together with the American groups cluster the farthest distance from the Europeans. The 2nd dimension separates African groups on one side and Oceanian groups to the other side from all other worldwide groups that appear centre. We then performed an AMOVA test to see how much of the total genetic variation provided by

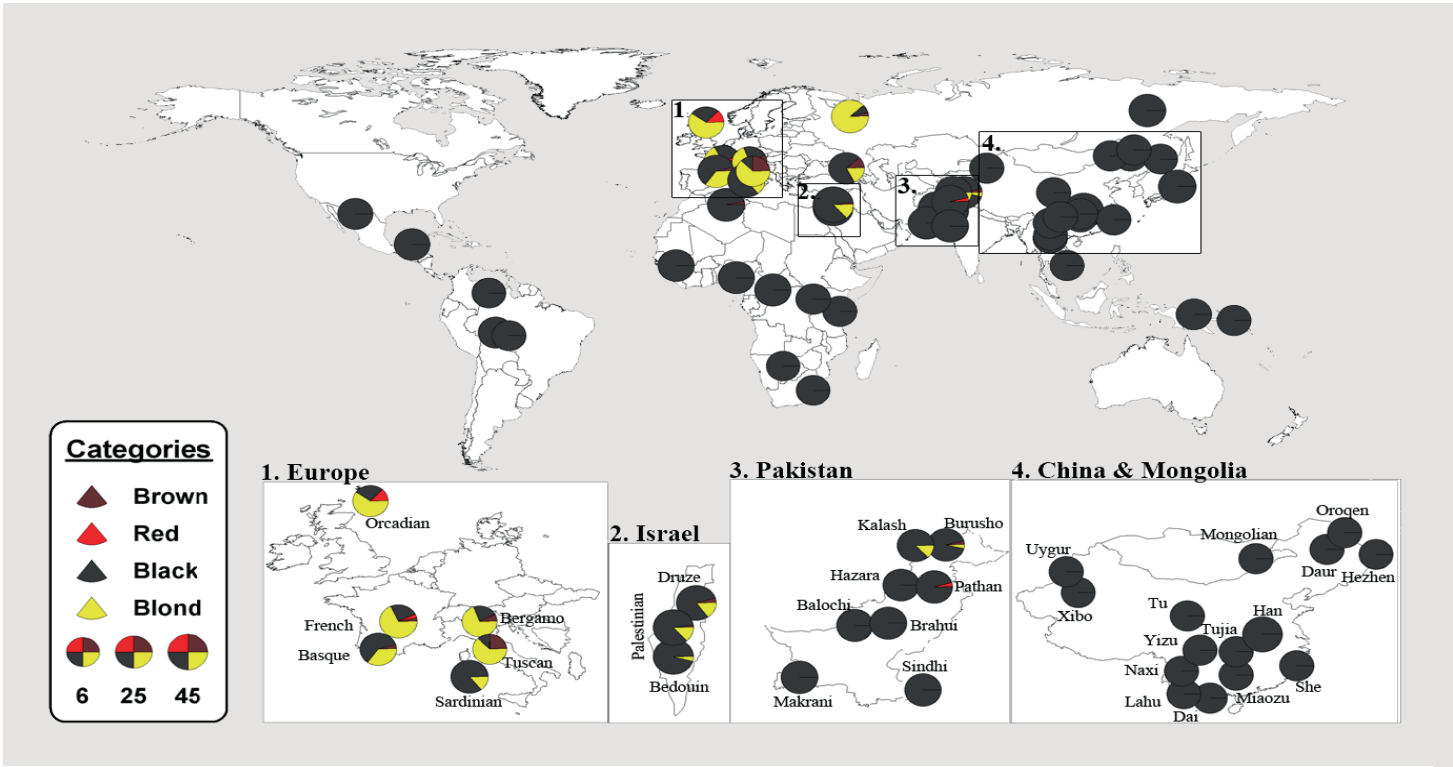

Figure 6: Worldwide depiction of the performance of the HIrisPlex system for hair colour prediction on the HGDP-CEPH H952 set of 945 individuals from 51 populations. No threshold is in place for prediction where the highest categorical p value (not taking into account shade) is deemed the predicted colour. There is a higher propensity of blond individuals predicted solely based on category p values within Europe, when using the guide from Figure 4, these numbers tend to decrease with an increase in brown/light brown predictions (data not shown). For worldwide eye colour prediction analysis using the IrisPlex system see Figure 5 of our previous IrisPlex paper [4] (Note that IrisPlex represents the eye colour part of HIrisPlex with the very same SNPs).

these 24 eye and hair colour predictive DNA variants is explained by geography when assigning the 51 populations into seven continental groups; Europe, Middle East, Africa, Central South Asia, East Asia, Oceania and America. A remarkably high variance proportion of 24.44% was estimated from 1100 permutations, which was highly statistically significant ($P < 0.000005$).

When separating the 51 populations into two groups i.e. Europeans and non-Europeans, we obtained a very similarly high variance proportion of 24.76% ($P < 0.000005$) from 1100 permutations. Grouping the 945 individuals according to their predicted hair colour categories

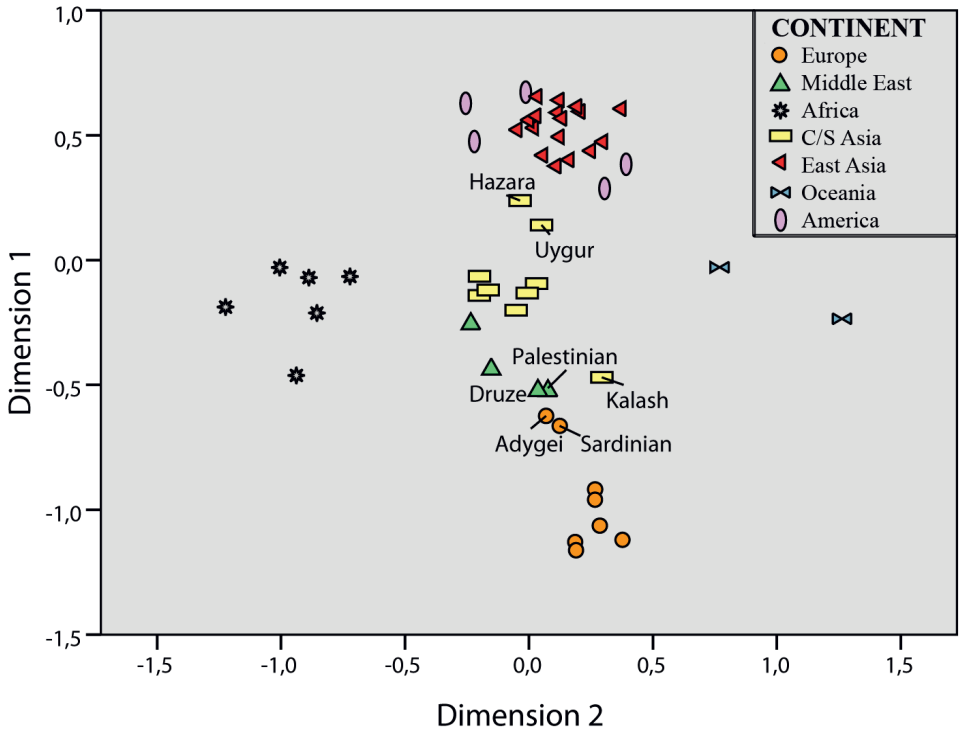

Figure 7: Two-dimensional plot from a non-metric multidimensional scaling (MDS) analysis of pairwise F_{ST} distances between the 51 worldwide HGDP-CEPH populations using the 24 HIrisPlex eye and hair colour DNA variants. Colour coding is according to geographic regions as provided in the legend. Populations in between two geographic clusters are given with names.

(black, brown, red and blond) resulted in an only slightly higher variance proportion of 29.79% ($P < 0.000005$) as expected for a European trait such as hair colour variation.

Motivated by this finding, we investigated a combined eye and hair prediction threshold to test if it may be possible to find out simply by means of HIrisPlex eye and hair colour probability strength if a brown-eyed and black haired individual originates from Europe or from a region distant to Europe. If successful, this would provide additional information to the sheer eye/hair colour prediction, as it may alleviate the potential need for ancestry testing in finding out more about an unknown crime scene sample donor / missing person. Obviously, a prediction with sufficiently high probability of blue or intermediate eye colour, as well as of brown, blond or red hair colour would already allow a conclusion that the person is of at least partial European descent. However, this is different for brown-eyed, black-haired predicted individuals as this phenotype combination occurs worldwide. The results of this non-European threshold assessment can be seen in Supplementary Figure 2 with the breakdown of population numbers shown in Supplementary Table 1. Our data demonstrate that it is indeed possible to predict that a brown-eyed, black-haired individual is likely to have non-European ancestry (excluding the nearby regions of Middle East and partly North Asia and America) using a threshold of >0.7 p for black hair and >0.99 p for brown eyes and the prediction accuracy based on our dataset is 86.5% (see Supplementary Table 1 for precise numbers).

We also investigated the worldwide allelic distribution of the 24 HIrisPlex DNA variants in the HGDP-CEPH samples separately for every DNA marker as shown in Supplementary Figures 3-5 (except for

rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs12896399 (*SLC24A4*), rs16891982 (*SLC45A2* (*MATP*)), rs1393350 (*TYR*) and rs12203592 (*IRF4*), as they can be found in Figure 4 of our previous publication on worldwide IrisPlex analysis [4]). Notably, none of the DNA variants provides such a strong degree of separation of Europeans versus Non-Europeans as *HERC2* SNP rs12913832 (see Figure 4 in [35], which is the highest ranked SNP in eye colour prediction [42] and also displays high probability values for hair colour prediction, except for red (Table 3). Although the *MC1R* variants displayed in Supplementary Figure 2(A) N29insA, (B) rs11547464, (D) rs1805008, (F) rs1805006, and Supplementary Figure 3 (G) rs1805007, (H) rs1805009, (I) Y152OCH which are all "high penetrance" *MC1R* variants as well as (K) rs111400 a "low penetrance" *MC1R* variant, all have a restricted European and surrounding areas distribution as expected given their role in red hair that is normally observed in individuals with European and nearby ancestry, they are all quite rare especially N29insA and Y152OCH. However the remaining *MC1R* variants included in HIrisPlex (rs885479, rs1805005, rs2228479) show a variable distribution within Europe and its proximate areas, as well as outside these regions, which may explain the very rare occurrence of red hair individuals outside of Europe and surrounding areas [59], or that their effect size is rather minor. Notably, both rs1805005 (Supplementary Figure 2(E), and rs2228479 (Supplementary Figure 3(J)) were grouped into the *MC1R_r* low penetrance group for red hair prediction in our previous publication [35] and require a combination of *MC1R* alleles before the red hair phenotype is displayed due to their minor contributions towards red hair, which would explain their distribution outside Europe as their red hair effect is more minor. Rs885479 (Supplementary Figure 2 (C)) was also deemed a

"low penetrance" SNP that is responsible for red hair colour production, but it seems to contribute to other hair colours as well as seen in its effect on the prediction model in Figure 2, where the largest effect by its minor allele contribution was towards the darkening of hair colour (brown-black) in comparison to its contribution towards red hair colour prediction. This SNP is also noted to have a skin colour contribution, especially related to the evolution of lighter skin colour in East Asians [60], which mirrors its worldwide allelic distribution as shown here. Another HIrisPlex SNP with a peculiar worldwide allele distribution is rs28777 in the *SLC45A2 (MATP*) gene (Supplementary Figure 3(L)), which reflects a pattern of European (and surrounding areas) versus Non-European differentiation due to its hair, in particular AA (black) v CC (red) colour effect, but also due to its assumed skin colour association [8]. Notably, its distribution is similar to rs1042602 in the *TYR* gene (Supplementary Figure 4(O)), which has been previously associated with normal hair colour variation and freckles [6]. Although it did not show significant association with hair colour in our previous paper [35], it did however provide an independent effect on hair colour prediction [35], which reflects this non-synonymous SNP's vital role in pigmentation. Rs683 (*TYRP1*) (Supplementary Figure 4(R)) also reflects a slight European v non-European pattern in terms of its TT genotype, which is present at a higher frequency within Europe and its surrounding areas than outside in which its counterpart allele GG is predominant. For the remaining SNPs, Supplementary Figure 4(M), rs12821256 (*KITLG*), (N) rs4959270 (*EXOC2*), (P) rs2402130 (*SLC24A4*), (Q) rs2378249 (*PIGU/ASIP*), although associated with hair colour in Europeans, there is no discernable pattern of allelic distribution worldwide.

Considerations on the practical use of the HIrisPlex system for hair and eye colour prediction

The HIrisPlex system is capable of simultaneously predicting the hair and eye colour of an individual from DNA. Practical recommendations for eye colour prediction using the HIrisPlex system follow those previously published for the IrisPlex system [19] as the very same 6 SNPs and the very same eye colour prediction model used in IrisPlex are also used in the HIrisPlex system when it comes to eye colour. To allow easy use of the HIrisPlex system in practical applications, and to take full advantage of our eye and hair colour genotype and phenotype database and its relevant parameters for model-based prediction, we provide with the present paper the HIrisPlex hair and eye colour prediction tool (Supplementary Table 2). This tool is a combined Excel macro specifically designed to manage both the eye colour and the hair colour prediction models in an easy-to-use fashion that allows interactive use. Users simply input the number of minor alleles (0, 1, or 2) of each of the 24 DNA variants included in the HIrisPlex assay and a probability value for black, brown, red and blond hair colour is produced based on the underlying hair colour prediction model, as well as separately the probability of light and dark hair colour shade, and separately the eye colour probabilities of blue, intermediate and brown based on the underlying eye colour prediction model. This tool replaces our previously provided Excel spreadsheet for eye colour prediction based on the IrisPlex system as it combines eye and hair colour prediction with the respective underlying database knowledge in one tool. For the most accurate interpretation of the categorical hair colour and hair shade prediction probabilities revealed from the Excel

macro prediction tool (Supplementary Table 2), we recommend to follow the hair prediction guide as shown in Figure 4.

As a working example of the tool, upon assessment of the 308 individuals used for model testing based solely on the highest probability category, we also assessed their hair colour prediction following the prediction guidelines set in this paper (Figure 4) as well as eye colour assessment following the guide set in the pan-European IrisPlex paper we published previously [19]. This reflects how the DNA prediction of both pigmentation traits would be performed in practice, with a final hair colour prediction being made to the case officer i.e. "the most probable hair colour is light blond", including the accuracy at which the system is able to predict the hair colour category based on current research (at present, based on our 308 individual test set), and the eye colour prediction would follow our previously published guidelines [6], i.e. the most probable eye colour result is brown above others (if this p value was >0.7p) at an accuracy of 94% based on a European dataset of over 3800 individuals." In Figure 8 we show four illustrative examples including eye and hair colour phenotypes from high-resolution photographs, the categorical eye and hair colour as well as hair shade probabilities as derived from HIrisPlex genotyping, and a summarizing statement of the prediction outcomes as may be used for reporting purposes (these individuals were not used in modelling).

Supplementary Table 3 provides the actual single grader (Polish) and self reported (Irish and Greek) hair and eye colour phenotype of the individual and includes the final prediction that would be produced with the HIrisPlex system for hair and eye colour. An accuracy of 60% correct prediction for both hair and eye colour together (measured as the

presence of an inaccurate prediction for either hair colour or eye colour) was achieved in this 308 model testing sample set. Expectedly, an increased number of individuals would be beneficial to test the accuracy of the HIrisPlex hair colour prediction model, especially from different countries in Europe other than Poland, Ireland and Greece that were involved in modelling to rule out any possible bias that may be present, which should be targeted in future studies. However, the relatively low percentage of correct combined eye and hair colour prediction in this test set is not only influenced by sample size but also by the different accuracies achieved for eye colour on one hand and hair colour on the other. For instance, in only 7% of the test individuals (all with intermediate eye and brown to black hair colours) were both pigmentation traits, eye and hair colour, predicted incorrectly.

When splitting-up the accuracies in this test set for the two pigmentation traits separately, hair colour alone was 76% correctly predicted using the prediction guide approach. Although different prediction accuracies were obtained for different hair colours as described above, the majority of the error lay in predicting a colour lighter than the physical phenotype, which can be attributed to the darkening of hair colour with age. Without having available biomarkers informative for the age-dependent hair colour change, we believe it will not be possible to dramatically reduce the prediction error currently obtained in such individuals. Consequently,

Figure 8: Four diverse examples of European individuals (A-D) illustrating the application of the HIrisPlex system including final outcome summaries as might be used for reporting purposes. Each individual depicts high-resolution hair and eye images, which display their actual eye and hair colour phenotypes. The eye and hair colour, as well as hair shade categories are shown with their respective probabilities as derived from HIrisPlex genotyping and input into the eye and hair colour prediction model, with the category displaying the highest probability per each trait highlighted. In relation to hair colour, the written statement underneath is completed by using the hair colour prediction guide described in this study including currently known prediction accuracies for a certain hair colour as based on the test set of 308 individuals from 3 European populations used in this study. Regarding eye colour the statement is completed by following guidelines for eye colour prediction described previously [19] using threshold assessment accuracies previously produced from a test set of >3800 individuals from 7 European populations [19]. None of the four individuals shown here were used for model building or model testing on eye and hair colour prediction.

basic research in the molecular biology of age-dependent hair colour changes is required to investigate whether such biomarkers can indeed be developed for future applications such as forensics.

For eye colour categories alone, 76% of individuals gave probabilities that were correctly predicted in this set without using a threshold, or 82% by applying the >0.7p threshold as we advocated before [19]. This overall estimate of eye colour prediction accuracy is strongly influenced by the intermediate/green category, which, with the currently available SNPs, is known to be by far the least accurately predictable eye colour category in relation to blue and brown. In fact, the majority (59%) of individuals in this test set that showed inaccurate eye colour prediction belonged to the intermediate category and only 14% intermediate (total n = 50) eye coloured individuals were predicted correctly. In contrast, and even without considering the previously suggested probability threshold of 0.7 p and omitting the phenotypic intermediate individuals, in only 8% of cases did the HIrisPlex system provide an incorrect prediction for individuals who had phenotypic blue eye colour and in only 18% of cases for individuals who had phenotypic brown eye colour in this set. This reflects an accuracy call rate of 88% (N = 258) for blue and brown eye colours alone in this test set, which could be increased to 94% (N=194) by applying the >0.7p threshold for correctly predicting the phenotypic blue and brown-eyed individuals within our set. Our previous IrisPlex study >3800 individuals from seven countries of different parts of Europe also provided an overall eye colour prediction accuracy of 94% for blue and brown using the >0.7p threshold [19]. This indicates that the eye colour accuracy when just considering blue and brown eye colour predictions is much higher than in the prediction of all

three categories, blue, brown and intermediate, mainly due to the fact that currently, DNA markers with the ability to strongly predict non-blue and non-brown eye colours are lacking and need to be established in future basic research.

Some of the individuals categorised as intermediate eye colour in fact carry green eyes and several DNA variants have been previously suggested to be informative for green eye prediction such as *OCA2* rs1800407 [7, 17]). Recently, Pneuman et al. [61] stated that green eye prediction with a high degree of accuracy is possible using specific genotype combinations i.e., A/G at rs12913832 plus T/T at rs12203592 designed combo 1, or G/G at rs12913832 plus C/C at rs16891982 designed combo 2. We were interested to see if we could improve the green eye prediction in our test set where we only achieve 5 correct intermediate/green (19%) predictions from the 27 phenotypic green eyed individuals considered. Using their guidelines, we found that combo 1 predicted only 2 of the 27 green individuals (8%), which is less than half of the ones correctly predicted by HIrisPlex, and wrongly predicted 3 blues as green. Combo 2 did not exist within this set of 308 individuals; hence, none of the remaining 25 green eyed individuals could be identified with this combo. Therefore, applying the approach of Pneuman et al. [61] to this data did not yield an improvement of green eye colour prediction in our test set. We believe, as we advocated before [4, 19], intensified basic research into the genetics underlying green eye colour is needed before better markers for green eye prediction in practical applications such as forensics can be provided.

CONCLUSIONS

The hereby introduced HIrisPlex system is capable of simultaneously predicting hair and eye colour phenotypes from DNA using a single 24-multiplex assay and a combined eye and hair colour prediction tool. The HIrisPlex genotyping assay is highly sensitive allowing successful genotyping down to at least 63 pg starting DNA, and is capable of successfully coping with degraded DNA due to fragment sizes of <160 bp. An on-going developmental validation study of the HIrisPlex assay will deliver additional characteristics relevant for forensic applications. The HIrisPlex hair colour prediction model and guide revealed on average individual-based hair colour prediction accuracies of 69.5% for blond, 78.5% for brown, 80% for red and 87.5% for black hair using our prediction guide approach. The HIrisPlex system provides reliable hair colour prediction independent from bio-geographic ancestry as we previously also showed for eye colour prediction and the IrisPlex system, which represents the eye colour prediction part of the new HIrisPlex system. HIrisPlex hair and eye colour prediction in practical applications is eased by providing a user-friendly Excel spreadsheet requiring not more than the input of the number of minor alleles of the 24 assay DNA variants. It produces individual probabilities for four hair colour categories (red, blond, brown, and black) and hair colour shade (light and dark) – used together and following the prediction guide approach we developed, this allows a more specific hair colour estimation than available from the categorical approach alone. This spreadsheet also delivers three eye colour categories (blue, intermediate, and brown) based on the previously developed and validated IrisPlex model. As an extra element with investigative value we demonstrate here that it is possible to infer bio-

geographic ancestry on the level of European (including nearby regions) versus non-European (excluding nearby regions) origin from the strength of HIrisPlex hair and eye colour probabilities for brown eyed and black haired individuals distributed worldwide (whereas non-brown eye colour and non-black hair colour *per se* indicate an origin in Europe, including nearby regions).

Current limitations of the HIrisPlex system are in accurately predicting hair colour in those individuals who underwent age-dependent changes that influenced category shifts (such as blond to brown) because of the current unavailability of biomarkers to indicate such a colour change, and in accurately predicting intermediate eye colours such as green because of the current unavailability of good DNA predictors for these non-blue and non-brown eye colours. Basic research for finding more appropriate bio-markers for these aspects is needed to overcome current limitations of DNA-based eye and hair colour prediction in the future. Furthermore, future research is needed on the biology and genetics of hair greying, and the development of informative bio-markers for its molecular prediction. Last but not least, and similar to our previous proclamation on eye colour [4, 19], we would like to emphasise here that only moving DNA-based hair (and eye) colour prediction from the current categorical level to a future continuous level, aiming to accurately predict all shades of hair (and eye) colour including age-dependent changes in early and in advanced ages, will provide the highest level of accuracy, as may be wished by the investigating authorities for forensic applications. Notably, such continuous prediction approach will also avoid the current uncertainties that come along with the interpretation variance of hair and eye colour categories by different receiving investigators, by being able to

provide them with actual colour hair and eye charts or printouts to be used for tracing an unknown person instead of a simplified colour category as possible for the time being.

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Supplementary Material can be found at
http://www.sciencedirect.com/science/article/pii/S1872497310000323
Or the **APPENDICES** of this Thesis.

CHAPTER 3.2

Developmental Validation of the HIrisPlex System: DNA-based eye and hair colour prediction for forensic usage

Draft prepared for submission to
Forensic Science International: Genetics

CHAPTER 4

APPLICATIONS OF DNA PHENOTYPING: EYE & HAIR COLOUR PREDICTION

CHAPTER 4.1

Human eye and hair colour variation is older than 5,300 years.

Submitted to Pigment Cell & Melanoma research

CHAPTER 4.2

Bona fide colour: DNA prediction of human eye and hair colour from ancient and contemporary skeletal remains.

Investigative Genetics 4(3) (2013)

ABSTRACT

Background: DNA analysis of ancient skeletal remains is invaluable in evolutionary biology for exploring the history of species, including humans. Modern human bones and teeth, however, are relevant in forensic DNA analyses that deal with the identification of perpetrators, missing persons, disaster victims, or family relationships. They may also provide useful information towards unraveling controversies that surround famous historical individuals. Retrieving information about a deceased person's externally visible characteristics can be informative in both types of DNA analyses. Recently, we demonstrated that human eye and hair colour can be reliably predicted from DNA using the HIrisPlex system. Here we test the feasibility of the novel HIrisPlex system at establishing eye and hair colour of deceased individuals from skeletal remains of various post-mortem time ranges and storage conditions.

Results: Twenty one teeth between 1 and approximately 800 years of age and 5 contemporary bones were analyzed using the HIrisPlex system of which all but three (88%) yielded the full 24 SNP HIrisPlex profile, therefore successfully allowing model-based eye and hair colour prediction. HIrisPlex analysis of a tooth from the Polish general Władysław Sikorski (1881-1943) revealed blue eye colour and blond hair colour, which was positively verified from reliable documentation. The partial profiles collected in the remaining three cases (two modern samples and a XIV century sample) were sufficient for eye colour prediction but did not allow hair colour prediction in one of them.

Conclusions: Overall, we demonstrate that the HIrisPlex system is suitable, sufficiently sensitive and robust to successfully predict eye and

hair colour from ancient and contemporary skeletal remains. Our findings therefore highlight the HIrisPlex system as a promising tool in future routine forensic casework involving skeletal remains, including ancient DNA studies, for the prediction of eye and hair colour of deceased individuals.

BACKGROUND

Skeletal remains represent a unique type of biological material. Due to their unique features, bones and teeth can be resistant to degradation and, depending on the environmental storage conditions, can provide a good source of DNA suitable for genetic analysis thousands of years after an organism's death. DNA analysis of ancient bones and teeth is used to explore various aspects of a species history including archaic and modern humans. For instance, the application of next generation DNA sequencing (NGS) technologies to ancient human bones has allowed the establishment of whole genome information on Neanderthals [1] and Denisovans [2]. These studies not only revealed that Denisovans represent a distinct archaic human taxon [2], which was impossible to conclude from the finger bone found as its only evidence, but also highlighted that genetic admixture likely occurred between both archaic human taxons and modern humans, respectively [1-4].

Contemporary bones and teeth are frequently used in dedicated forensic laboratories dealing with missing person identification cases, including disaster victim identification. Although DNA extraction from skeletal remains is very complex and time consuming with limited possibilities for automation, in forensic science teeth and bones constitute the most suitable sources of DNA for individual identification from heavily decomposed bodies. Forensic DNA analysis of contemporary bones and teeth typically concerns autosomal short tandem repeats (STRs, or microsatellites) for direct match identification with antemortem samples of the same individual, or via STR profile similarities with close putative relatives [5,6]. Mitochondrial (mt) DNA and Y-chromosomal DNA analysis

also represent two genetic marker systems, that allow profile matching not only with close but also with distant putative maternal and paternal relatives, respectively [7]. Furthermore, there seems to be continual interest in the DNA analysis of remains from famous historical individuals as seen with studies about the last Russian Tsar Nikolaus II (1868-1918) and his close family [8-11], the Polish astronomer Nicolaus Copernicus (1473-1543) [12], the Italian poet and founder of humanism Francesco Petrarca (1304-1374) [13], or perhaps even the evangelist Luke [14] to name but a few examples.

One interesting aspect of ancient and modern skeletal analysis, which has been largely overlooked thus far, is the reconstruction of the deceased person's appearance. In ancient DNA analysis the prediction of particular externally visible characteristics (EVCs) not reflected in skeletal features such as eye and hair colour, is the only way to get insights into how extinct species may have looked. In contemporary skeletal analysis, such as forensic cases, EVC prediction from DNA can be useful in providing leads in police investigation to reveal the identity of a deceased person if other and more direct means (such as STR, mtDNA and Y-DNA profiling) are non-informative. Recent years have started seeing increased success in DNA-based EVC prediction, a field sometimes referred to as Forensic DNA Phenotyping (FDP) or DNA intelligence [15,16]. From all human externally visible characteristics (besides sex), pigmentation traits particularly eye and hair colour are currently the most promising EVCs for DNA prediction. This is due to limited genetic complexity coupled with limited environmental impact that has lead to accumulative success in exploring the genetic basis of eye and hair colour via candidate gene and genome-

wide association studies [17-22]. From these association studies, highly predictive eye and hair colour DNA markers have been identified [23-33].

Previous ancient DNA studies have suggested that Neanderthals may have had light skin and reddish hair colour due to a particular non-synonymous variation in *MC1R* [34]. Genetic variation in *MC1R* indeed causes light skin colour and red hair colour in modern humans [35], albeit via different DNA variants than found in Neanderthals in that study. Recently, whole genome sequencing (WGS) data from bones of Neanderthals, Denisovans, and modern humans from different time periods have been established using NGS technologies and in part have been explored for the prediction of pigmentation (and other visible and non-visible) traits [36-38]. However, the establishment of WGS data from bones, though shown to be feasible via NGS, still comes with a stiff price tag not easily affordable by most laboratories. Furthermore, WGS represents a type of data overkill when one is only interested in specific traits determined by a limited number of genes such as eye and hair colour. Lastly, WGS comes with the disadvantage that if the overall genome coverage is low (which often is the case in aDNA NGS/WGS studies) the quality for the particular single nucleotide polymorphisms (SNP) genotypes required for prediction can be low with consequences for the final trait prediction. What therefore would be welcomed are more dedicated and targeted approaches allowing the retrieval of high quality EVC information such as eye and hair colour from ancient and modern human skeletal remains in an effective manner such as multiplex analyses..

Previously, we developed and forensically validated the IrisPlex system for reliable and accurate DNA prediction of blue and brown eye

colour, which includes a sensitive single multiplex assay of 6 highly eye colour predictive SNPs and an eye colour prediction model based on genotype and phenotype data of many thousands of individuals from various regions in Europe [24-26]. An independent test dataset of >3800 individuals from 7 European sampling sites have shown that the IrisPlex system provides on average 94% individual-based accuracy for blue and brown eye colour prediction, respectively [26]. In an earlier study, some pigmentation associated SNPs had been combined in a multiplex assay and tested successfully on ancient bone samples [39]. However, the predictive value of some of the SNPs considered in that study is low and lower than the SNPs that only became available via subsequent studies. In particular, they typed only 2 of the 6 best eye colour predictors included in the IrisPlex assay. Recently, a highly sensitive single multiplex genotyping assay has been developed, together with model-based tools supported by large genotype and phenotype databases, allowing parallel prediction of eye and hair colour from a DNA sample [40]. This so called HIrisPlex system targets 24 eye and/or hair colour predictive DNA polymorphisms (including all 6 IrisPlex SNPs) and provides individual prediction probabilities for eye and hair colour categories as well as hair colour shade categories [40]. As shown recently, this approach allows the prediction of hair colour with average individual-based prediction accuracies of 69.5% for blond, 78.5% for brown, 80% for red and 87.5% for black hair colour on an independent test set of >300 individuals from 3 European sampling sites [40]. Furthermore, it was previously shown by analyzing a worldwide sample set (HGDP-CEPH) that IrisPlex/HIrisPlex eye and hair colour prediction works reliable independent of knowledge on bio-geographic

ancestry [24,40]. In the present study we test the feasibility of predicting eye and hair colour of deceased individuals from DNA analysis of ancient and contemporary teeth and bones using the novel HIrisPlex system.

METHODS

Bone and teeth samples

The specimens subjected to DNA analysis comprised of 21 teeth and 5 bones. Samples S1- S3 were collected from modern human remains approximately 1-2 years after death as estimated from medico-legal and criminalistic examination. S4 was a tooth collected from modern remains of an incomplete skeleton that rested in soil; it was impossible to estimate an approximate age of the remains but anthropological examination showed they are seriously decayed. Serious degradation was also present in cases S5 to S10, which were modern human remains found in a cave. S5 was a fragment of femur, S6 was a fragment of tibia, S7 was a fragment of humerus, S8 was a fragment of another humerus, S9 was a tooth collected from a mandible and S10 was a part of a skull; they were all found in one location in a cave situated in Dolina Będkowska Landscape Park near Kraków. S11 was a tooth collected from exhumed remains of General Władysław Sikorski; the exact date of his death is known (1943) and thus we know that the analyzed tooth was 69 years of age. S12-S23 were samples collected from skulls which in 1942 were subjected to bleaching and afterwards kept in a museum in Austria until 1998. S24 was a tooth of a mysterious woman from Benedictine crypt in Tyniec near Kraków, which according to historical evidence comes from the XII-XIV century. S25 and S26 were teeth picked up from skeletons found in the St. Andrew Church in Kraków and dated based on historical markers to come from the XIV

century.

Precautions taken to avoid contamination

Multifarious efforts were undertaken in the laboratory in order to prevent contamination. Full protective clothing and separate working localities for extraction, amplification, and SBE reaction setup were used. All DNA extractions were done in a separate room reserved for low template DNA samples. DNA extraction and PCR were performed in clean room facilities with Laminar Air Flow benches equipped with HEPA filters. All working areas and equipment were regularly UV-irradiated and cleaned with bleach. In total 10 negative extraction controls were included. Each presented case was subjected to separate DNA extraction, except for samples S12-S23 where three negative extraction controls were used. Negative controls were also included for each PCR amplification performed. All samples were first subjected to STR analysis which showed no traces of DNA admixture that could indicate contamination. Moreover, in order to exclude any possibility of internal contamination, STR (AmpFℓSTR® NGM™ kit) and HIrisPlex profiles were determined for all 10 members of the laboratory staff. No match was found for any of the samples analysed in both STR and HIrisPlex profiles.

DNA extraction

DNA was isolated using standard organic extraction protocols. Before extraction, bone material comprising of teeth or fragments of femurs was subjected to a purification and decontamination procedure. Samples were treated with 15% bleach, repeatedly shaken with 70% ethanol and

distilled water, and while drying subjected to UV irradiation. The entire tooth or bone fragments were then pulverized using FreezerMill 6750 (Spex CertiPrep) and subjected to the extraction procedure. When available 3 g (in case of tooth samples 1-1.5 g) of bone powder were incubated overnight in a water bath set at 56°C with 3mL of buffer composed of 0.5 M EDTA and 10% SDS with addition of 225 µL proteinase K (10 mg/mL) and 120 µL 1 M DTT. The samples were then subjected to double extraction with a phenol-chloroform-isoamyl alcohol (Sigma) and concentrated after extraction using Amicon Ultra 4 – 30k columns and Microcon 100 (Millipore) to the final volume of 60-70 µl in all cases. Negative extraction controls were used to check the purity of the used chemicals and consumables.

DNA quantification

DNA concentration was assessed on a 7500 Real Time PCR system using ***Quantifiler***™ Human DNA Quantification Kit (Applied Biosystems), following the protocol recommended by the manufacturer.

Genotyping with the HIrisPlex assay

Multiplex genotyping was performed as described in detail recently [40]. The HIrisPlex assay relies on simultaneous PCR amplification followed by simultaneous SNaPshot primer extension (or minisequencing) of 24 DNA polymorphisms previously established with predictive value on human eye and hair colour, namely N29insA, rs11547464, rs885479, rs1805008, rs1805005, rs1805006, rs1805007, rs1805009, Y152OCH, rs2228479, rs1110400 – all from the *MC1R* gene , rs12913832 from *HERC2*, rs12203592

from *IRF4*, rs1042602 and rs1393350 from *TYR*, rs4959270 from *EXOC2*, rs28777 and rs16891982 from *SLC45A2*, rs683 from *TYRP1*, rs1800407 from *OCA2*, rs2402130 and 12896399 from *SLC24A4*, rs12821256 from *KITLG* and rs2378249 from *ASIP*. All samples were genotyped at least twice.

Prediction of eye and hair colour with the HIrisPlex system

Eye colour inference from the genotyping results of the 6 SNPs (the same as used in IrisPlex) was obtained via model-based prediction using a database of thousands of Europeans and a convenient excel macro published previously [24,26]. Hair colour inference from the genotypes of 22 SNPs was obtained via model-based prediction using a database of >1500 Europeans with an excel macro and following a prediction guide that not only considers hair colour categories but also hair colour shade for the final assessment as introduced recently [40].

RESULTS AND DISCUSSION

Using a standard organic extraction protocol, we purified genomic DNA from 21 teeth aging between 1 year and approximately 800 years (XII century) and 5 contemporary bones. All samples were subjected to multiple genotyping using the HIrisPlex assay and to model-based eye and hair colour prediction from the combined HIrisPlex genotypes using large genotype/phenotype databases described elsewhere [24,26,40]. It is also worth emphasising that in studies of skeletal remains, unlike some other human characteristics, pigmentation cannot be concluded from basic anthropological research and thus reliable DNA prediction of eye and hair colour shall be particularly useful. The ascertained HIrisPlex genotypes for all 24 DNA polymorphisms and all 26 samples analysed are presented in Table 1. The derived eye and hair colour probabilities, the predicted eye and hair colours and the accuracy probability values of predicted eye and hair colours for all 26 individuals are provided in Table 2. Although in all but one of the samples analysed the phenotypic eye and hair colour of the deceased individuals analysed were unknown, the previously obtained accuracy estimates from large numbers of individuals with known phenotype and genotype information provide reasonable confidence that the eye and hair colour phenotypes predicted in this study match the true phenotypes of the individuals before death. However, because previous studies showed that the estimated probabilities and attached accuracies are higher for eye than for hair colour categories, more confidence can be expected in eye colour over the hair colour prediction results obtained here.

Contemporary samples

A number of contemporary forensic DNA identification cases remain unsolved because of no match between the evidence STR profile and a reference DNA profile from a suspect or from the forensic DNA database search. Similarly, a number of missing person cases including disaster victim identification (DVI) cases remain unsolved because no STR profile match with the antemortem samples, or because no informative STR profile similarities with known putative relatives can be obtained. In all such cases EVC information inferred from the scene sample, including skeletal remains, may be valuable for the continuing investigation. Representing typical forensic casework samples, we included three teeth collected from partially or completely skeletonized bodies of approximately 1-2 years post mortem age. S1 was a cadaver and S2 was a skeleton, both found in small villages in different regions of Poland in the open soil. S3 was a cadaver found inside a building located in Kędzierzyn-Koźle, a small city in southern Poland. The teeth were previously subjected to standard forensic identification using STR and mitochondrial DNA profiling. Complete STR profiles (***NGM***™ PCR Amplification ***Kit*** or ***Identifiler***® PCR Amplification Kit) were obtained in each case indicating reasonably good preservation of nuclear DNA. The amelogenin gender test revealed that skeletons S1 and S3 were males and S2 a female, corroborating results of the anthropological investigation of the skeletons (data not shown). Complete HIrisPlex profiles were determined for all three samples (Table 1). For S1-S3 the highest probability (p) was obtained for brown eye colour (S1=0.490, S2=0.552 and S3=0.539, with accuracies S1=87.5%, S2=91%, and S3=90.4%, Table 2). In cases S1 and S3 hair colour

was predicted as dark brown (accuracy 78.5%) and in case S2 hair colour was predicted as dark blond/ brown (accuracy 78.5%, Table 2). Samples S1 and S3 were not associated with any reference material and all the genetic data including eye and hair colour inference may be used in further investigating these cases. Sample S3 was associated with a reference sample from a putative close relative but in the course of genetic analysis the assumed hypothesis of a relationship was denied.

Occasionally, human skeletal remains are discovered coincidentally by workers or sightseers. They may be of old or young post mortem age. Sample S4 came from the human remains found in the field near Sieradz in central Poland. Anthropological examination revealed that the bones belong to a woman of about 40 years of age at the time of death. The skeletal remains may have rested in the soil for a very long time as they appeared to be seriously decayed. Two teeth were taken from the maxilla for DNA extraction. A partial NGM STR profile was determined (seven markers were genotyped successfully) and the positive amelogenin result confirmed the female origin suggested from the anthropological investigation. In this case the level of DNA concentration was critical as ***Quantifiler***™ Human DNA Quantification Kit indicated merely 7 pg/µl of human DNA. As template DNA concentration in the HIrisPlex reaction equaling approximately 30 pg was below the genotyping accuracy threshold of approximately 60 pg as previously established [40], genotyping was repeated three times. Results for all but one (86insC *MC1R*) HIrisPlex SNPs were obtained successfully. Blue eye colour was inferred with high probability (p = 0.919) and accuracy (p = 97.4), (Table 2). Blond hair color was inferred with a probability of 0.864 and light hair

Sample ID	Sample/ postmortem age	Starting template DNA [ng]	Genotype																					
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
S1	T/CR	0.6	C	G	C	C	G/T	C	C	G	C	G	T	A	G	A	C/A	C/T	G	G	A	C/T	T	T	C	G/T
S2	T/CR	1.7	C	G	C/T	C	G	C	C	G	C	G	T	A	G	A	C/A	C	G	G	A	C/T	T	T	C/T	G/T
S3	T/CR	1.2	C	G	C/T	C	G	C	C	G	C	G	T	A	G	G/A	C/A	C/T	G/T	G	G	C/T	T	G	C/T	G/T
S4	T/C	0.3	-	G	C	C/T	G	C	C	G	C	G	T	A	G	A	C/A	C	G/T	G	A	C	T	G/T	C	T
S5	B/C	U[a]	C	G	C	C	G	C	C	G	C	G	T	C	C	A	C/A	C/T	G/T	G	A	C	T	T	C	G
S6	B/C	0.3	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C	C	G/T	G	A	T	T	G/T	C	T
S7	B/C	0.15	-	G	C	C	G	C	C	G	C	G	T	C	C	A	C/A	C/T	G/T	G	A	C	T	T	C	G
S8	B/C	U	C	G	C	C	G	C	C	G	C	G	T	C/A	G/C	A	C/A	C	G	G	A	C/T	T	G	C	G/T
S9	B/C	0.5	C	G	C	C	G	C	C	G	C	G	T	C/A	G/C	A	C/A	C/T	G	G	A	C	T	T	C/T	G/T
S10	B/C	0.1	C	G	C	C	G	C	C	G	C	G	T	C/A	G/C	A	C	C	G	G	G/A	T	T	G/T	C/T	G/T
S11	T/2WW	0.6	C	G	C	C/T	G	C	C	G	C	G	T	A	G	A	A	C	G/T	G	A	C	T	T	C	T
S12	T/2WW	2	C	G	C	C/T	G	C	C	G	C	G	C/T	A	G	A	C	C	G	G	A	C	T	G/T	C/T	G/T
S13	T/2WW	0.3	C	G	C	C/T	G	C	C	G	C	G	T	A	G	A	A	C	G	G	A	C/T	T	T	C	G
S14	T/2WW	0.2	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C/A	C	T	G	G/A	C	T	G	C/T	T
S15	T/2WW	1.2	C	G	C	C	G	C	C	G	C	G	T	A	G	A	A	C	G/T	G	G/A	C	T	G	C	G
S16	T/2WW	0.1	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C/A	C	G	G	A	C	T	T	C	T
S17	T/2WW	0.8	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C/A	C	G	G	A	C/T	T	G/T	C/T	G/T
S18	T/2WW	2.8	C	G	C	C/T	G	C	C	G	C	G	T	A	G	A	C/A	C	G/T	G	A	C	C/T	G/T	C/T	G/T
S19	T/2WW	0.8	C	G	C	C	G	C	C	G	C	G	T	A	G	A	A	C	G	G	A	C	T	T	C	T
S20	T/2WW	0.4	C	G	C	C	G	C	C	G	C	G	T	A	G	G/A	C	C	G/T	G	A	C/T	T	G/T	C/T	G/T
S21	T/2WW	0.3	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C/A	C	G	G	A	C	T	G/T	C/T	T
S22	T/2WW	0.7	C	G	C	C	G	C	C	G	C	G	T	A	G	G	A	C	G	G	G	C	C/T	G	C	T
S23	T/2WW	1.3	C	G	C	C	G	C	C	G	C	G	T	A	G	A	C/A	C	G/T	G	A	C/T	T	G/T	C	T
S24	T/XII	0.03[b]	C	G	C	C	G	C	C	G	C	G/A	T	A	G	A	C/A	C	G	G	A	C/T	T	G/T	T	G/T
S25	T/XIV	0.01[b]	-	G	C	C	-	C	T	G	C	-	T	A	G	A	C/A	C	T	G	G	C	T	G	C/T	G/T
S26	T/XIV	0.16	C	G	C	C	G	C	C	G	C	G/A	T	A	G	A	C/A	C	T	G	A	C	T	G/T	C	G/T

[a] - U - DNA concentration was not determined due to complete inhibiton of ***Quantifiler***™ Human DNA Quantification Kit reaction.

[b] - DNA concentration may be higher as significant inhibition of ***Quantifiler***™ Human DNA Quantification Kit reaction was observed. DNA markers are shown in the following order from 1 to 24: 1-N29insA, 2-rs11547464, 3-rs885479, 4-rs1805008, 5-rs1805005, 6-rs1805006, 7-rs1805007, 8-rs1805009, 9-Y152OCH, 10-rs2228479, 11-rs1110400, 12-rs28777, 13- rs16891982, 14- rs12821256, 15-rs4959270, 16- rs12203592, 17- rs1042602 , 18-rs1800407, 19-rs2402130, 20-rs12913832, 21- rs2378249, 22-rs12896399, 23-rs1393350, 24- rs683. Samples S5 and S7 come from the same individual. In the column Sample/postmortem age: T indicates tooth and B indicates bone. CR – indicates contemporary and relatively fresh specimen (1-2 years postmortem age); C – indicates contemporary but older than CR; 2WW – indicates samples from the World War 2; XII and XIV – indicate centuries assumed from historical evidence.

Table 1. HIrisPlex genotyping results together with sample age and starting DNA amount for the 26 samples tested.

colour shade with a probability of 0.976 so that light blond is assumed as most likely hair colour. However, red hair cannot be excluded because of the missing ins86A *MC1R* genotype. Although this DNA variant (indel) is very rare in the general European population (allele observed 4 times among more than 1000 samples, data not shown), its impact on red hair colour is very strong.

Samples S5-S10 came from human remains discovered in one of several caves located in the landscape park Dolina Będkowska near Kraków (Poland). Anthropological examination indicated that the revealed 13 skeletal elements, including pelvis, femurs, humerus, ribs, vertebras, mandible comprising two teeth and an incomplete skull, may belong to 6 individuals. The same mtDNA and STR profiles were ascertained in the femur (S5) and the humerus (S7) indicating that these bones likely belong to a single female skeleton (data not shown). Overall genetic analysis showed that in fact all remains came from only 5 individuals, two females (S5/S7 and S9) and three males (S6, S8 and S10). However, only a partial STR profile was determined in S7 showing more serious degradation compared with S5. Both types of bone (femur and humerus) are usually considered a good source of DNA and thus the observed difference may be coincidental. Complete 24 SNP HIrisPlex profiles were generated for all samples except for S7 where again the ins86A *MC1R* genotype was missing. The obtained data show that from all 24 HIrisPlex polymorphisms indel position 86insA in *MC1R* seems most affected by suboptimal DNA concentration and/or DNA degradation. The S7 profile was also inconsistent among three repeated analyses carried out in terms of rs4959270.

Sample	Probability values of hair colour categories	Probability values of hair colour shade	Inferred most likely hair colour	Accuracy probability value of predicted hair colour based on a >300 European test set	Probability values of eye colour categories	Inferred most likely eye colour	Accuracy probability value of predicted eye colour based on a >3800 European test set
S1	Brown 0.367 Red 0.002 Black 0.499 Blond 0.133	Light 0.268 Dark 0.732	Dark Brown	78.5%	Blue 0.317 Int. 0.193 Brown 0.490	Brown	87.5%
S2	Brown 0.246 Red 0.001 Black 0.326 Blond 0.427	Light 0.655 Dark 0.345	Dark Blond/Brown	78.5%	Blue 0.306 Int. 0.142 Brown 0.552	Brown	91%
S3	Brown 0.496 Red 0.001 Black 0.406 Blond 0.098	Light 0.215 Dark 0.785	Dark Brown	78.5%	Blue 0.190 Int. 0.271 Brown 0.539	Brown	90.4%
S4	Brown 0.064 Red 0.048 Black 0.025 Blond 0.864	Light 0.976 Dark 0.024	Light Blond	69.5%	Blue 0.919 Int. 0.048 Brown 0.033	Blue	97.4%
S5	Brown 0.251 Red 0.000 Black 0.729 Blond 0.020	Light 0.020 Dark 0.980	Black/ Dark Brown	87.5%	Blue 0.706 Int. 0.117 Brown 0.177	Blue	94%
S6	Brown 0.227 Red 0.000 Black 0.636 Blond 0.136	Light 0.171 Dark 0.829	Dark Brown	78.5%	Blue 0.002 Int. 0.026 Brown 0.972	Brown	99%
S7	Brown 0.282 Red 0.000 Black 0.690 Blond 0.028	Light 0.030 Dark 0.970	Black/Dark Brown	87.5%	Blue 0.706 Int. 0.117 Brown 0.177	Blue	94%
S8	Brown 0.246 Red 0.000 Black 0.594 Blond 0.160	Light 0.196 Dark 0.804	Dark Brown	78.5%	Blue 0.024 Int. 0.083 Brown 0.892	Brown	95.6%
S9	Brown 0.324 Red 0.001 Black 0.538 Blond 0.136	Light 0.212 Dark 0.788	Dark Brown	78.5%	Blue 0.911 Int. 0.057 Brown 0.032	Blue	97.4%
S10	Brown 0.153 Red 0.000 Black 0.829 Blond 0.017	Light 0.016 Dark 0.984	Black	87.5%	Blue 0.001 Int. 0.016 Brown 0.983	Brown	99%
S11	Brown 0.076 Red 0.037 Black 0.035 Blond 0.852	Light 0.964 Dark 0.036	Light Blond	69.5%	Blue 0.950 Int. 0.030 Brown 0.020	Blue	99%
S12	Brown 0.044 Red 0.090 Black 0.049 Blond 0.817	Light 0.969 Dark 0.031	Light Blond	69.5%	Blue 0.937 Int. 0.041 Brown 0.022	Blue	97.4%
S13	Brown 0.180 Red 0.025 Black 0.119 Blond 0.676	Light 0.883 Dark 0.117	Dark Blond/ Light Brown	69.5%	Blue 0.229 Int. 0.128 Brown 0.643	Brown	91.9%
S14	Brown 0.157 Red 0.005 Black 0.072 Blond 0.766	Light 0.907 Dark 0.093	Light Blond	69.5%	Blue 0.899 Int. 0.066 Brown 0.035	Blue	95.6%

Table 2. HIrisPlex-based eye and hair colour prediction results for the 26

S15	Brown 0.168 Red 0.004 Black 0.064 Blond 0.765	Light 0.927 Dark 0.073	Light Blond	69.5%	Blue 0.870 Int. 0.076 Brown 0.053	Blue	95.6%
S16	Brown 0.089 Red 0.002 Black 0.054 Blond 0.856	Light 0.951 Dark 0.049	Light Blond	69.5%	Blue 0.950 Int. 0.030 Brown 0.020	Blue	99%
S17	Brown 0.221 Red 0.001 Black 0.263 Blond 0.514	Light 0.696 Dark 0.304	Dark Blond/Brown	78.5%	Blue 0.207 Int. 0.161 Brown 0.632	Brown	91.9%
S18	Brown 0.083 Red 0.090 Black 0.024 Blond 0.802	Light 0.969 Dark 0.031	Light Blond	69.5%	Blue 0.937 Int. 0.041 Brown 0.022	Blue	97.4%
S19	Brown 0.075 Red 0.002 Black 0.039 Blond 0.883	Light 0.968 Dark 0.032	Light Blond	69.5%	Blue 0.950 Int. 0.030 Brown 0.020	Blue	99%
S20	Brown 0.246 Red 0.001 Black 0.183 Blond 0.570	Light 0.727 Dark 0.273	Dark Blond/Brown	78.5%	Blue 0.207 Int. 0.161 Brown 0.632	Brown	91.9%
S21	Brown 0.089 Red 0.002 Black 0.054 Blond 0.856	Light 0.951 Dark 0.049	Light Blond	69.5%	Blue 0.937 Int. 0.041 Brown 0.022	Blue	97.4%
S22	Brown 0.155 Red 0.008 Black 0.053 Blond 0.783	Light 0.934 Dark 0.066	Light Blond	69.5%	Blue 0.870 Int. 0.076 Brown 0.053	Blue	95.6%
S23	Brown 0.218 Red 0.002 Black 0.248 Blond 0.532	Light 0.696 Dark 0.304	Dark Blond/Brown	78.5%	Blue 0.150 Int. 0.140 Brown 0.711	Brown	94%
S24	Brown 0.202 Red 0.002 Black 0.301 Blond 0.495	Light 0.695 Dark 0.305	Dark Blond/Brown	78.5%	Blue 0.277 Int. 0.179 Brown 0.543	Brown	90.4%
S25	-	-	-		Blue 0.899 Int. 0.066 Brown 0.035	Blue	95.6%
S26	Brown 0.131 Red 0.007 Black 0.078 Blond 0.784	Light 0.918 Dark 0.082	Light Blond	69.5%	Blue 0.919 Int. 0.048 Brown 0.033	Blue	97.4%

Probability estimation and final prediction of eye colour was done according to Walsh et al. 2011 [24] and eye colour accuracy estimation according to Walsh et al. 2012 [26].
Probability and accuracy estimation and final prediction for hair colour and hair colour shade was done according to Walsh et al. 2012 [40].

Table 2 continued. HIrisPlex-based eye and hair colour prediction

From the HIrisPlex profile ascertained in sample S5 we could further conclude that drop-out (rs4959270C/A -> A) occurred in this position in

sample S7 (observed twice among three repetitions) keeping with the hypothesis that indeed both samples derive from the same individual. Inferred eye and hair colours for S5 and S7 are blue and black/dark brown, respectively, with highly similar probabilities and accuracies agreeing with the idea that they were derived from the very same individual. Notably, strong PCR inhibition prevented determination of DNA concentration in samples S5 and S8 using ***Quantifiler***™ Human DNA Quantification Kit (Table 1). Nevertheless, genotyping was possible in both samples showing that the HirisPlex assay is indeed insensible to inhibition similarly to commercial STR kits used for human identification purposes. Inferred eye colour was blue for S9 (probability 0.911, accuracy 97.4%) and brown for S6, S8 and S10 (probabilities 0.972, 0.892, and 0.983, respectively; accuracies 99%, 95.6% and 99%, respectively). Inferred hair colour was dark brown for S6, S8 and S9 (accuracy 78.5%) and black for S10 (accuracy of 87.5%).

Samples from World War II

DNA analysis from skeletal remains may help to solve various controversies concerning historical figures. As an illustrative example we analysed tooth S11 (Figure 1) collected from the corpse of General Władysław Sikorski.

Figure 1. The photograph of the analysed tooth of General Władysław Sikorski (World War II).

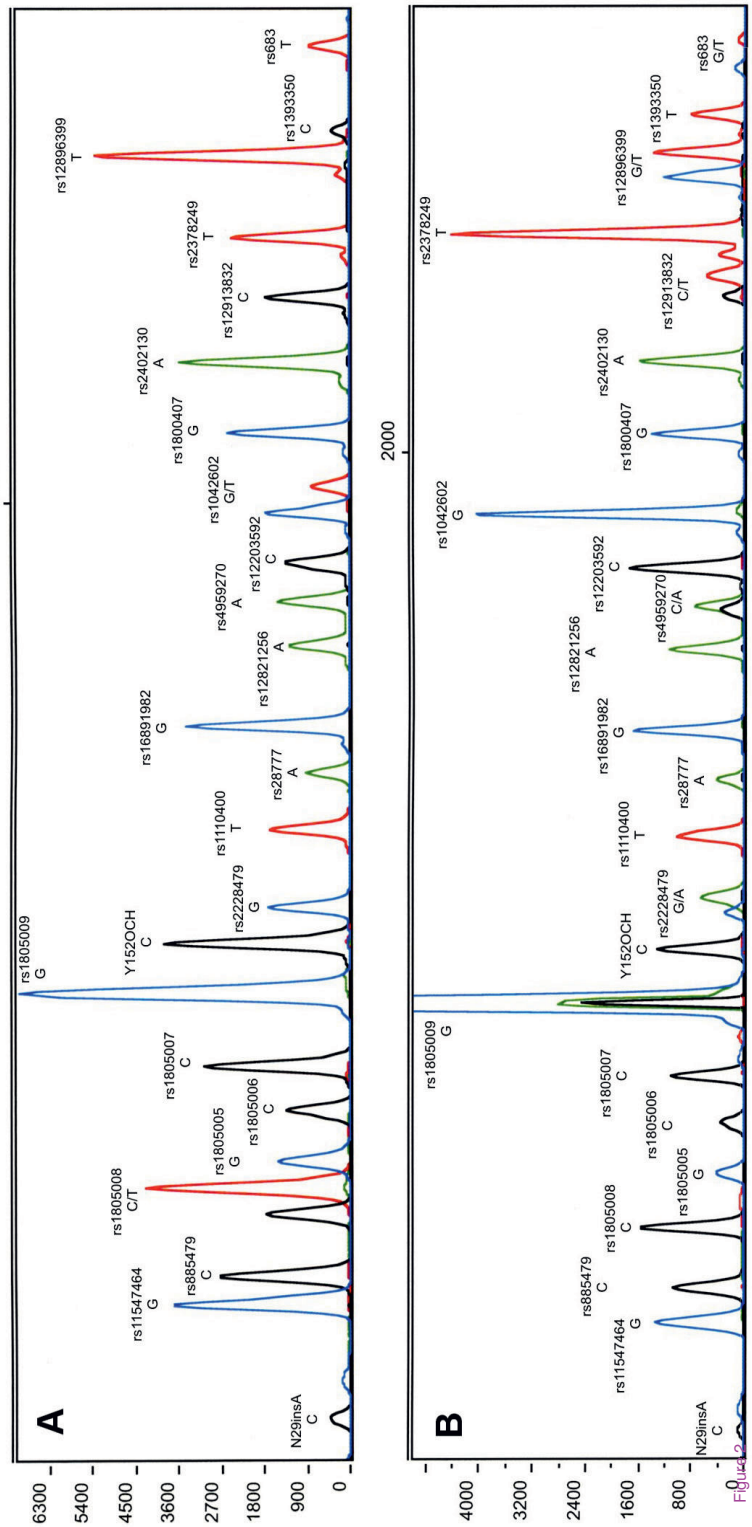

Figure 2. HIrisPlex SNP electropherograms obtained from two selected teeth samples. A - a tooth of the Polish General Władysław Sikorski (World War II), B – a tooth of a mysterious woman from the Benedictine Abbey in Tyniec, Poland (XII-XIV century). Peaks reflect alleles determined in the HIrisPlex loci, which are identified on electrophoregrams with rs numbers or in two cases with position in amino acid chain and mutation type (insertion N29insA, nonsense mutation Y152OCH). Pull-up peaks resulted from increased fluorescence observed at position rs1805009 in Panel B.

During World War II Sikorski was Commander-in-Chief of the Polish Armed Forces and also was Prime Minister of the Polish government in exile. Sikorski died in an airplane crash at Gibraltar in 1943. His body was buried in cemetery in Newark (UK) and after exhumation in 1993 was finally placed in the crypt of the cathedral of the Royal Castle on Wawel Hill in Kraków (Poland). Since the hypothesis that an accident had caused the airplane crush at Gibraltar was questioned, in 2008 Władysław Sikorski was again exhumed and the body was thoroughly examined [41].

Previous mitochondrial DNA analysis of the tooth was crucial for identification of General Sikorski's remains, but a complete Identifiler STR profile and a complete Yfiler Y-STR profile were obtained too, indicating reasonably good DNA preservation [42]. A complete HIrisPlex SNP profile was generated and is shown in Figure 2.

The HIrisPlex model predicted blue eye colour with high probability ($p = 0.950$) and high accuracy (99%) as well as blond hair colour ($p = 0.852$) and light hair colour shade ($p = 0.964$) so that light blond can be assumed as most likely hair colour (accuracy 69.5%). It is worth noting that in this particular case the eye/hair colour predictions could be confronted with known data about Władysław Sikorski's appearance. Although, colour photographs of the General were unavailable and all known colour portraits were painted many years after his death and thus may not be considered most reliable, the fact that Władysław Sikorski indeed had blue eyes and blond hair, as predicted via HIrisPlex, was obtained from historical scripts [43].

DNA analysis of recent human skeletal remains can also be helpful in the identification of war victims. As examples, teeth S12-S23 were collected from persons who were killed during World War II in a prison

located in Poznań (Poland). In 1942 fourteen skulls were sent to the Natural History Museum in Vienna and at that time they were exhibited as examples of "sub-human" skulls. In 1998 these skulls were returned to Poland, and the Institute of National Remembrance ordered examination of these human remains. In all 12 cases besides mitochondrial DNA data, complete Minifiler STR profiles and partial Yfiler Y-STR profiles were obtained (data not shown) indicating some level of genomic DNA fragmentation. All 12 skulls were included in this study for teeth sampling. In all 12 cases complete HIrisPlex SNP profiles were generated (Table 1) and the eye and hair colour prediction results are presented in Table 2. Among these 12 individuals, blue (probabilities 0.87-0.95) and brown eye colours (probabilities 0.632-0.711) were inferred with high accuracies (91.9%-99%) as well as light blond, light blond / dark blond and dark blond / brown hair colours (accuracies 69.5-78.5%), as may be expected from individuals of (most likely) Polish European ancestry.

Medieval samples

Analysis of DNA extracted from old bone samples is often a very challenging task due to very low amounts of DNA achieved, which additionally is very often subject to heavy degradation. Sample S24 represents a controversial case from the Benedictine Abbey in Tyniec near Kraków. During the works undertaken in the crypt of the St. Peter and Paul church belonging to the Abbey, 17 skeletons of alleged abbots were found. The burial was dated to the period of the XII-XIV century. Unexpectedly, the anthropological examination revealed that two skeletons may be of female origin, which indeed was confirmed by DNA analysis (data not shown), while only male monks were expected. One of

the two DNA samples was sufficiently preserved to enable analysis of other nuclear markers (data not shown) and was used here for HIrisPlex analysis. The mysterious woman was predicted with dark blond/brown hair (accuracy of 78.5%) and brown eyes (accuracy of 90.4%), (Table 2 and Figure 2, panel B). The somewhat stronger peak imbalance relative to the sample shown in the electropherogram panel A can be explained by the lower DNA quality of this medieval sample and resulting preferential amplification. Consequent higher signals observed at rs1805009 (above 8000 RFU) led to elevated pull-up peaks. These artifacts however did not interfere with the phenotype interpretation from the obtained genotype data.

Samples S25 and S26 came from two skeletons revealed during conservation works conducted in the Church of St. Andrew in Kraków in 2011. The church of St. Andrew was built between 1079 and 1098 representing a great example of the Romanesque style. Two medieval skeletons were found under the floor between the chancel and the nave of the church. Based on historical markers the grave was dated to originate from the XIV century. Further anthropological examinations indicated that the S25 male died at the approximate age of 60 whereas the S26 male was approximately 75 years old at the time of death. It is alleged that the skeletons belong to members of Tęczyński family representing noble Polish magnates of medieval times. The tooth collected from the deeper burial (S25) was found to be seriously affected by decay, which was reflected by very low DNA concentration (3 pg/µl) and incomplete autosomal and Y chromosome STR profiles (NGM and Yfiler). Complete mtDNA HVI and HVII profiles were generated in both teeth (data not shown). From these data it was possible to conclude that

both skeletons are of male origin and are unrelated in both maternal and paternal lines. From the partial HIrisPlex profile ascertained from S25 we successfully inferred blue eye colour (p = 0.899, accuracy of 95.6%), but hair colour could not be inferred because of missing genotypes at three DNA variants (N29insA, rs1805005, rs2228479). The sample S26 revealed a prediction of blond hair colour (p = 0.784) together with light hair colour shade (p = 0.918) concluding that the individual had light blond hair (accuracy of 69.5%). Eye colour prediction of S26 revealed blue eyes (p = 0.919, accuracy of 97.4%), (Table 2).

The HIrisPlex system was initially designed to enable degraded DNA analysis with considering short amplicons (less than 160 bp for all amplicons) [40]. Here we showed that indeed HIrisPlex performs successfully in degraded DNA from skeletal remains of various ages and storage conditions. However, also under such design the possibility of allelic drop-outs and drop-ins, which have been described as typical phenomenon associated with analysis of low template DNA samples, cannot be eliminated completely. Allele drop-outs, which are explained by stochastic effects, may lead to false homozygote genotypes whereas allele drop-ins are explained by minute contaminations. Both effects may affect final results of DNA-based human identification [44,45]. Allelic drop-outs and drop-ins can also have a practical impact on EVC prediction for example HIrisPlex based eye and hair colour prediction. However, the final effect depends on the particular SNP involved, as different SNPs have different impact on the final eye and hair colour prediction. Such influence may be particularly strong for strong DNA predictors such as the IrisPlex/HIrisPlex marker rs12913832 in the *HERC2* gene, which strongly determines blue/brown eye colour. From the homozygote CC/GG

genotype blue eye colour is predicted while the heterozygote CT/GA genotype most often indicate hazel/light brown eye colour [26]. A similar situation occurs in case of several DNA variants in the *MC1R* gene such as N29insA, rs1805007 and rs1805008 that have high penetrance among red hair colour individuals. All samples analysed here were genotyped at least twice and in most cases the results were consistent. Inconsistencies were observed mainly at two DNA variants, the insertion/deletion polymorphism N29insA (first position in the assay) and the polymorphism C/A rs4959270 (15. position in the assay). N29insA was most sensitive to suboptimal quality/quantity of template DNA. The peak height is generally lower compared to the other SNPs in the HIrisPlex assay and did not reach detection limit settled at 50 RFU 5 times in the course of repeated analyses. It should be also mentioned that in 3 cases (S4, S7 and S25) this polymorphism remained undetermined (Table 1). For rs4959270 we most probably observed drop-out in two samples (S14 and S17). Table 2 shows the prediction results under the assumption of the presence of the heterozygous state CA/GT in both cases as is presented in Table 1. This discrepancy has little influence on the prediction values but affects the conclusion for S14 in terms of hair colour (light blond/dark blond for homozygote C vs. light blond for heterozygote C/A). Although drop-out was assumed as more probable, drop-in can not be completely ruled out. Notably, these two DNA variants were also discussed by Walsh et al. as more sensitive than all other HIrisPlex SNPs to template DNA quantity [40]. In one of two the three genotypings performed on sample S13 drop-ins were also identified at rs2402130G (position 19 of the assay), rs12896399 G (position 22) and rs683T (position 24). We assumed that these signals reflect minor contamination because they were detected only once and at

significantly lower level than other peaks in the HIrisPlex profile of this sample. Negative extraction and PCR controls were clean in case of STR analyses in this sample while in some HIrisPlex negative controls 1-3 peaks at a very low signal level were detected. This effect seems to be stochastic and there is no particular DNA variant in the HirisPlex assay, which can be pointed as more prone to such minor contamination.

The results obtained here provide further evidence that quality of DNA templates from bone material depends not only on the storage time but more so on environmental conditions affecting the decomposition of the remains. Low temperature and low humidity are known to prevent DNA degradation [46] allowing successful DNA analysis even after tens of thousands of years [1]. Here we show that a complete 24 SNP HIrisPlex profile was obtained from an XII century sample (S24) collected from a skeleton that rested in relatively favourable conditions in a church. On the other hand, a partial profile was obtained from a relatively young contemporary sample (S17) that was found in open soil. However, it is difficult, as had been outlined previously [46] to draw generalizing conclusions from such data. For instance, a different XII century sample stored under expectedly similar conditions also excavated from inside a church (S25) yielded a partial HIrisPlex profile.

Obviously, special care is needed when EVC prediction is performed for samples containing DNA in suboptimal quantity and/or quality. Previous preliminary sensitivity testing of the HIrisPlex assay [40] provided an approximate threshold of 60 pg, above which allelic drop-out and drop-in were not observed. In the present study, two samples were analysed from an estimated starting template DNA amount of less than 60 pg, of which one (S24 ~30 pg) revealed a full HIrisPlex profile allowing

both eye and hair colour prediction, while the other (S25 ~10 pg) lacked genotypes at three DNA variants, which – because of the particular DNA variants involved in the drop-out - did allow for eye colour but not hair colour prediction. However, the concentration estimates established for these two samples may not be realistic as both extracts displayed signs of DNA inhibition from Quantifiler RT-PCR measurement. In any case, application of the HIrisPlex assay from low quality/quantity DNA samples, such as DNA extracted from skeletal remains or touched objects, should be accompanied with appropriate rigor meeting recommendations settled for low template DNA samples [47].

CONCLUSIONS

It can be anticipated that DNA prediction of EVC will soon become more widely used in genetic studies of human remains in evolutionary, anthropological, and forensic investigations. The recently introduced HIrisPlex system [40] provides a convenient molecular tool for simultaneous prediction of eye and hair colour categories from DNA. As demonstrated here, the HIrisPlex system is sufficiently sensitive and robust to enable successful analysis of bones and teeth of various ages including medieval (and expectedly older) samples. HIrisPlex can be successfully implemented in a forensic DNA laboratory equipped with the standard layout for DNA identity testing and specialized on DNA extraction from skeletal remains, and likewise in any dedicated ancient DNA laboratory. With the current study we aim to encourage the anthropological and forensic genetic community to use the HIrisPlex system for DNA-based eye and hair colour prediction in future evolutionary, anthropological studies and for forensic case work involving skeletal remains.

Abbreviations

NGS, next generation DNA sequencing; mtDNA, mitochondrial DNA; EVC, externally visible characteristic; STR, short tandem repeat (polymorphism); Y-STR, Y chromosome STR; FDP, forensic DNA phenotyping; WGS, whole genome sequencing; aDNA, ancient DNA; SNP, single nucleotide polymorphism; HGDP-CEPH**,** human genome diversity cell line panel Foundation Jean Dausset-CEPH Paris; RT-PCR, real time polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl

sulfate; DTT, dithiothreitol; DVI, disaster victim identification; indel, insertion-deletion polymorphism

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WB and MK conceived and designed the study, provided resources, interpreted results, and wrote the manuscript. JDB carried out the genotypings and elaborated results. EP participated in genotyping data analysis. SW carried out the prediction of phenotypes from genotypes and helped in manuscript writing. TK and HG provided the samples and helped to draft case description parts of the manuscript. All authors read and approved the final manuscript.

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CHAPTER 5

GENERAL DISCUSSION

In this thesis, DNA variants highly associated with human pigmentation traits were explored for their contribution towards accurate prediction systems for human externally visible characteristics. The main objective of the thesis was to build upon previous exploratory knowledge concerning DNA variants involved in eye and hair colour, and to unite them in the creation of useful DNA prediction systems for eye and hair colour. These systems consist of laboratory assays together with functional statistics that are applicable in forensic DNA phenotyping. This chapter formulates results from the overall thesis in a successive fashion, combining what was achieved with a further discussion on other methodologies and the knowledge we gained during this research for future investigative undertakings.

MAIN FINDINGS

At the time research for this thesis began (2009), the field of pigmentation genetics was well underway, with many groups focused on finding the genes responsible for pigmentation traits in humans. However with the work presented in this thesis, this has now led to the application of all this knowledge and data into DNA phenotyping. Publications from our group, and in particular the work presented here, helped guide this wave of research, with eye and hair colour being at the forefront, and led to the beginning of human appearance trait prediction as we know it today.

1. DNA Phenotyping: Eye colour prediction

For several years, intensive studies have led to an increase in understanding the genetic basis of human eye colour, from genome-wide association and linkage analysis to candidate gene studies [1-11]. In the

beginning of this thesis, with its core statistical fundamentals taken from epidemiological approaches in prediction design, findings behind a large population-based eye colour prediction analysis [12] performed by this group were made available for development. This led to the accomplishments I presented in **Chapter 2.1**; namely the creation of the IrisPlex system. IrisPlex is a single multiplex genotyping system for the 6 currently known most informative eye colour SNPs, which can reliably predict blue and brown eye colours with high levels of accuracy when combined in a prediction model. The system consists of an assay and a prediction model. The test assay is extremely sensitive allowing successful analyses from trace amounts of biological material down to 31 pg of DNA, approximately 6 human cell equivalents, using genotyping technology that relies on equipment widely available in the forensic community, a core fundamental in its design. A visual representation of this 6-SNP-eye colour prediction model is illustrated on newly generated data from 40 individuals with phenotypic information, where image ordering was solely based on prediction probabilities as opposed to phenotype information, and it gives an applied look at how the model performs (see Figure 2 in chapter 2.1). An insightful look into the worldwide distribution and genetic diversity associated with eye colour variation in Europeans using these 6 SNPs on 51 worldwide populations from the HGDP-CEPH set is also described, which finally relays qualms on the need for prior ancestry testing for eye colour prediction. Supporting evidence suggests that correct interpretation of the prediction outcomes from this test do not require additional genetic ancestry testing as it is unlikely that the combination of SNPs used here cannot detect the existence of non-brown eye colour outside Europe. Apart from the production of a system that is

capable of predicting an individual's eye colour from a sample of DNA, this ancestry-free aspect is a core finding in this article.

In **Chapter 2.2**, I show how the IrisPlex assay portion of the system was evaluated by running it through vigorous internationally accepted standard assessments implemented by the Scientific Working Group on DNA Analysis Methods (SWGDAM) [13]. As a result, the developmental validation of the IrisPlex assay, as part of the IrisPlex system, fulfils all SWGDAM guidelines in terms of species specificity, sensitivity, stability, and mixture studies, reproducibility, precision and accuracy, casework samples and population studies. The assay surpasses many genotyping assays currently used in the forensic context in terms of sensitivity, with 31 pg minimum DNA input. It is capable of producing accurate results from simulated casework samples that include blood, semen, saliva, hair, and trace DNA samples, including extremely low quantity samples. It also produces consistent results in both internal and external laboratory investigations with extreme ease of use and reliability. In conjunction with the human eye colour prediction model, which was described in the overall IrisPlex system in **Chapter 2.1** and further described in the next chapter, the IrisPlex system was officially made ready for use in any accredited forensic laboratory for aiding DNA intelligence investigations. In **Chapter 2.3**, I present convincing empirical proof that the IrisPlex model, the second component of the IrisPlex system which was previously developed by Liu *et al.* [12] using genotype and phenotype data from 3804 Dutch Europeans [12], is extremely reliable for the accurate prediction of blue and brown eye colour to a level of >94%. This information was calculated on analyses from >3800 individuals from seven centres across Europe with large variations in phenotype and

genotype data. This chapter examines the model as a whole, from the break-down of eye colour across Europe in these centres that follows a north to south gradient decrease in blue eye colour and subsequent increase in brown colour, to the definition of what is termed blue and brown eyes. Further statistical assessment of eye colour prediction also quells questions that refer to the reliability of using one country (The Netherlands from which the IrisPlex model was first prepared [12]) as acceptable for use in Multinomial logistic regression (MLR) modelling of eye colour prediction. For this, the total number of 3800 individuals from the seven European sites representing seven countries was used to create a new model. There was no discernible improvement seen. Lastly as prediction probability estimates for eye colour represent all three categories for the individual's final prediction, there are certain circumstances where probability values will be quite similar and the 'highest probability value representing the final prediction' may encounter difficulties. Therefore a full assessment of an acceptable threshold was carried out, taking a look at every result to yield a threshold level that provided a balance between specificity and sensitivity of the model with a final probability value > 0.7 being determined as optimal. This assessment is also the guide for which a report can dictate the accuracy of their new prediction, based on a European set of 3800 individuals. Overall the combination of **Chapter 2.1, 2.2 and 2.3** is a complete evaluation of the IrisPlex system as a whole, which really establishes its place as a tool in the forensic community and in DNA phenotyping for eye colour. Most notably, the scientific and technological achievements described in chapters 2.1, 2.2, and 2.3, in conjunction with earlier work by our group on ascertaining the most eye colour predictive

DNA markers and initially developing a prediction model, led to the progression of legislative changes within the Dutch government. This allowed the use of the IrisPlex eye colour prediction system in Dutch forensic casework as of May 2012.

Chapter 2.4 takes a new direction in eye colour prediction and brings the definition back to its roots from simply assigning eye colour phenotype variation to colour categories and instead allowing a more detailed definition of what eye colour is. Up to this chapter, the thesis was focused on categorical eye colour prediction, an approach that by nature simplifies phenotype diversity by combining similar eye colours into groups. Here, however, I describe a new quantitative approach to eye colour phenotyping and assess its impact on genetic association and DNA prediction. Quantitative eye colour was first investigated by Liu *et al.* [14], in the participation of the first genome wide assessment of eye colour using quantitative measurements. Before this, genetic studies on human eye colour were all based on categorical trait information, on a scale of blue, green or intermediate, and brown eye colour [1, 3, 4, 6, 8]. In reality as we know, there are numerous versions and collections of colour within the iris, which seem to exist on a more continuous grade from the lightest shades of blue to the darkest shades of brown [15]. Liu *et al.* [14] measured human eye colour using a transformed scale of new values from the Hue (H) and Saturation (S) spectrum of colour on digital images from thousands of Dutch Europeans. H measures the variation in colour spectrum and S measures variation in colour intensity. It is important to note that the methodology behind this paper, which was performed during the time of this thesis, was to try an alternative form of representing colour, in particular, colour within the iris. Several points on

the iris were used and an average was taken for these newly defined H and S values to be represented on the spectrum. These values were then used as new phenotypes and hence, a new GWAS study was performed. With this method, 17 predictors were found that significantly explained the trait variance, which was 48.87% of the H variance and 56.3% of the S variance using the Rotterdam Study sample set of >5900 individuals. This includes epistatic interactions between these SNPs that were also proposed to increase prediction accuracy of this quantitative trait. Hence this redefinition of colour was able to improve the power of finding new genetic associations with quantitative rather than categorical eye colour. This in turn led to an increase in power for prediction of eye colour quantities based on H and S with the addition of these new SNPs. This paper had proven that fine-resolution phenotyping was a better alternative to an increase in sample size that is used rather often in current GWA studies in humans as a way to improve results. Triggered by the success of this first quantitative eye colour study, in **Chapter 2.4**, we reconsidered the definition of quantitative eye colour. Despite the colour of the iris, in all its various forms, the number of melanocytes does not differ, but it is the quality and quantity of the two types of melanin in the cytoplasm that determines the observed colour [16]. In the new approach I describe in **Chapter 2.4**, the notion of melanin amount was considered and a system was developed that allows the measurement of the amount of eumelanin, pheomelanin and no visible melanin (pigment) within high-resolution digital eye imagery. Eumelanin, is a highly compact pigment, packed in ovoid eumelanosomes [17], which absorbs nearly the full light spectrum and appears as dark-brown to black. Pheomelanin in contrast is a more spread out pigment and is perceived as yellow to red [17, 18]. The

absence of melanin means that light is mostly reflected by the stroma of the iris and eye colour becomes perceived as grey to blue through the Tyndall scattering [19, 20]. In comparing the colour spectrum observed using HS values versus the new melanin amount measurements on the subset of individuals (n=3087) from our >3800 set with high quality imagery from **Chapter 2.3**, we see a much better separation of irises based on colour using this new approach. By using the 12 SNPs identified by Liu *et al*. [21] that are involved in continuous eye colour variation using a HS scale, and combining them into a quantitative prediction model, we could directly compare the observed variance using both methods (HS v Melanin amount). This comparison showed clear improvements in strength of association but also in the amount of eye colour variation explained by these DNA variants when applying the new melanin quantification approach. For H the variance observed was 49% and S variance was 66% using the subset of 3087 individuals as presented in **Chapter 2.3.** On a comparative scale, using the new melanin quantification approach, the variance observed with the same set of individuals was non-melanin amount at 74%, eumelanin at 57% and pheomelanin at 44%. The increase in associated variance seen with this new approach can be explained by its subject matter, it is measuring the two types of melanin, which is a biologicial phenomenon. In previous approaches, iris colour is measured, however this is based on the wavelengths of light that are scattered within the eye, therefore as a biological function it is more difficult to measure. We also found several epistatic effects between these 12 SNPs using this new phenotyping approach, which also suggests that a combination of these effects into a prediction model, including independent SNP effects, would increase the

models prediction accuracy substantially.

From **Chapter 2.4**, one can see that the new quantification method improves eye colour fine-phenotyping, which results in enhanced genetic association and DNA prediction using previously identified and associated SNPs. It can be expected that when this new approach is applied in a new GWAS, it has great potential in the identification of previously unrecognised eye colour genes and predictive DNA markers.

2. DNA Phenotyping: Hair colour prediction

With the progress of eye colour prediction and the development of systems such as IrisPlex, predicting externally visible characteristics (EVC) came under the magnifying glass, and the question asked was what would come next? As eye and hair colour run under quite similar biological and genetic pathways, the next logical step was looking at hair colour and the ability to predict this trait by using DNA variants previously implicated in human hair colour variation. As exemplified for eye colour [12], it is important to consider a large number of hair colour associated SNPs in large population studies to assess the order and rank of their impact, both individually and combined, to predict hair colour. This was done in several types of studies using different methods. Valenzuela *et al*. [22] evaluated 75 SNPs from 24 genes that were previously implicated in hair, skin and eye colour. In that study, samples from various bio-geographic origins were used, including populations outside of Europe (and its surrounding areas). That study found that a combination of three SNPs (rs12913832 (*HERC2*), rs16891982 (*SLC45A2*) and rs1426654 (*SLC24A5*)) gave the best prediction for light and dark hair colour [22]. It is important to note that for a study investigating a European trait such as

hair colour, it is vital to only use Europeans in evaluating the predictive value of each hair colour associated SNP. Using global samples, as done by Valenzueala *et al.,* [22] includes the risk of assigning a hair (or eye) colour predictive effect to a SNP that in reality shows an ancestry effect. This scenario is avoided completely when solely concentrating on a European dataset. In the study performed by Branicki *et al.* [23], an evaluation of 46 hair colour associated SNPs from 13 genes for population-based prediction of hair colour using a European data set was undertaken. They highlighted a set of 13 DNA markers (2 *MC1R* combined marker sets and 11 single DNA markers) from 11 genes (*MC1R, HERC2, OCA2, SLC45A2 (MATP), KITLG, EXOC2, TYR, SLC24A4, IRF4, PIGU/ASIP* and *TYRP1*) as the most informative hair colour predictors. This set provided a high degree of population-based, prevalence-adjusted prediction accuracy (that utilises an AUC assessment as similar to eye colour prediction by Liu *et al.* [12]) with estimates of 0.93 for red, 0.87 for black, 0.82 for brown, and 0.81 for blond hair colour, where 1 means completely accurate prediction. These high levels of population-based prediction accuracy led to the development of the HIrisPlex system I described in **Chapter 3.1**.

The HIrisPlex system is made up of a sensitive single multiplex assay together with two accurate prediction models, one for eye colour and the other for hair colour and shade. Using 24 DNA variants, this system has the ability to simultaneously predict both eye and hair colour of an individual from a sample left at a crime scene. Displaying accuracies of >94% for eye colour category prediction as seen in **Chapter 2.1**, together with accuracies of 79% on average for hair colour categories as seen in Chapter 3.1, it represents a valuable DNA prediction system and the first to combine two predictable appearance phenotypes

simultaneously. For the eye colour portion of the tool, it combines information from all IrisPlex model assessments [12, 21, 24] in its determination of eye colour prediction. For the hair colour portion of the tool, **Chapter 3.1** describes re-modelling on a higher number of individuals relative to what was initially used by Branicki *et al.* [23] (n=1243 versus n=385 [23]), due to the incorporation of additional samples from other countries within Europe, Ireland, Greece and Poland. This inclusion led to a better-rounded model based on representatives from around Europe, with Poland displaying lighter phenotypes, Greece displaying darker phenotypes and Ireland displaying a mixture of both with increased numbers of *MC1R* mutations. The development of the model also catered for the effect of 24 independent variants as opposed to the model proposed by Branicki *et al*. [23] where *MC1R* variants were grouped into two sets, the high penetrance and low penetrance group. This independent modelling allowed the incorporation of single MC1R mutations and their smaller effects towards other hair colour category predictions (i.e. brown hair colour). Also with the addition of hair colour shade, this provides a more precise prediction model. When measured on a test set of > 300 individuals, its accuracy is 79% on average, with blond at 69.5%, brown at 78.5%, red at 80% and black at 87.5%. Analyses on worldwide samples from the HGDP-CEPH set also show that HIrisPlex is reliable at hair colour prediction on a worldwide scale independent of biogeographic ancestry knowledge. It is even possible to predict non-European ancestry versus European ancestry with an accuracy of > 86.5%, using a combination of eye and hair colour probability thresholds of ≥ 0.99 and ≥ 0.7, respectively, which also provides additional intelligence to an investigation.

To test if the HIrisPlex assay can also pass all required SWGDAM guidelines for use worldwide, in **Chapter 3.2** it undergoes vigorous testing quite like those seen in **Chapter 2.2** for the IrisPlex assay. The minimum DNA input necessary for the HIrisPlex assay to produce complete 24 SNP profiles was down to 63 pg, the assay passes each requirement in terms of species specificity, sensitivity, stability, mixture studies, reproducibility, precision and accuracy, casework samples and population studies, exceeding regulatory standards. In this chapter I also release information on the online HIrisPlex prediction tool, capable of predicting eye and hair colour from full and partial profiles of the 24-variant HIrisPlex assay.

3. Applications of DNA Phenotyping: Eye and Hair colour

The real test of a tool is in its application and how useful and accurate it can be. Up to now in this thesis, I have described the application of the IrisPlex and HIrisplex systems for eye and hair colour prediction as being useful in the context of forensics. The use of these tools facilitates the production of a physical appearance description, in this case eye and hair colour, from genetic data such as DNA obtained from a crime-scene sample. Although it is not expected to replace conventional STR DNA profiling that is quite fixed in the forensic setting, it does add considerable information termed 'DNA intelligence' to an investigation. Therefore its application is deemed useful and informative, and in some cases, will be the only lead to explore if conventional forensic applications fall short i.e. no STR profile match to a suspect or any individual from a criminal database. However, forensics is not the only

field where this pigmentation application may find its use. In **Chapter 4,** I show how the eye and hair colour prediction systems can be applied to answer evolutionary, anthropological and historical questions. The field of biological anthropology looks upon our past to understand where we have come from, how we looked, what changed in our appearance and how we adapted to our environment. Using a pigmentation prediction tool such as the HIrisPlex allows us to phenotypically predict the appearance of our ancestors based on their genetic data using knowledge from individuals in the present. It has the ability to satisfy our curiosity as we are able to imagine what our ancestors may have looked like and not only does this application satisfy the inquisitive mind, but it also provides physical evidence of time points involved in our adaptive evolution. This information has the capacity to influence evolutionary genetics in the search for positive selection forces that have led to human adaption such as those in pigmentation. Furthermore, performing DNA phenotyping analyses on ancient samples such as the Neanderthal that have also been carbon-dated allow a concrete time-stamp to be included in simulated data, allowing a more accurate estimation of a particular genes selective power. It also allows the pigmentation appearance prediction of an individual that died a substantially long time ago from remains that do not provide physical evidence of pigmentation traits such as bones and teeth. **In Chapter 4.1,** I take knowledge gathered from HIrisPlex data across modern day Europe and the world (as described in **Chapters 2.1, 2.3 and 3.1**) to reveal the eye and hair colour predictions of ancient samples from published data evidence. HIrisPlex SNP data was inferred from publically available sequences of the Neanderthal, Denisova and the

Tyrolean Iceman. Following guidelines on the use of sequence data, the Iceman obtained a high probability of 0.972 for brown eye colour while probabilities for the other eye colours were very low (P_{blue}=0.002, $P_{intermediate}$=0.026), and using an adjusted HIrisPlex hair colour prediction model (23 SNP model) we obtained a black hair colour probability of 0.64 together with a brown hair probability of 0.307 while the probabilities for other hair colours were very low (P_{blond}=0.052, P_{red}=0.001). From this we could conclude that the Iceman had brown eyes and dark brown hair. However, upon examination of the probabilities and the increase in variation as opposed to the ancient samples of the Neanderthal (0.994 p brown eye colour) and the Denisova (0.994 p brown eye colour, 0.88 p black hair colour (0.117 p brown hair)), also reflecting the ancestral phenotypes and genotypes in human humans, it was clear that an increased level of variation at these pigmentation DNA variants was present in the Iceman. As the evolution of light skin colour has been researched extensively, an adaption to UV radiation has been postulated with a time-stamp of 30,000 years ago during the out-of-Africa migration with more selective European-specific alleles starting between 11,000 – 19,000 years ago [25]. Therefore it was necessary to account for the skin colour variation effect in the Iceman as it is more likely that he had lightly pigmented skin colour [26]. However, even taking the skin colour effect into account, eye and hair colour variation was still significant for several of the derived alleles present in the Iceman. This added evidence to our theory that eye and hair colour variation arose before 5300 years ago i.e. the time period when the Iceman lived. Furthermore, additional results from the *HERC2 SNP* rs12913832, the DNA marker that displays the

strongest association with and predictive for derived light eye and hair colours in contemporary humans, from newly analysed ancient DNA samples within Europe showed that its variation was already present and established at least 4,950 cal BP. Taking these findings together, this chapter concludes with propositions that eye and hair colour variation arose well before 5,300 years ago, the first data-supported timestamp to exist.

In **Chapter 4.2**, I continue to describe the application of eye and hair colour phenotyping to anthropological and historical issues, which are also of forensic relevance due to the investigation of degraded DNA samples often dealt with in forensic DNA analysis. In this chapter, I present the HIrisPlex analysis of DNA samples from contemporary and ancient (up to 800 years old) human remains. This chapter highlights the use of the HIrisPlex assay as a sensitive and robust tool to use on aDNA samples extracted from bones and teeth (including medieval samples). As pigmentation cannot be concluded from basic anthropological research, reliable DNA prediction of eye and hair colour using HIrisPlex can be used to predict an eye and hair colour phenotype from deceased remains, including those from ancient times. Results produced in this chapter using the HIrisPlex system highlight how a phenotyping approach can be used on individuals (remains) from our past, even historical figures. Among other samples, it describes the analysis of General Sikorski's remains, a prominent historical figure in Polish history, from the extraction of his DNA to the application of DNA phenotyping. The HIrisPlex data, confirmed with documented reports, show that he had blue eyes and blond hair, substantiating the reliability of the HIrisPlex prediction system as a whole. It is important to note however, that in the case of aDNA work,

the success of DNA phenotyping methods such as HIrisPlex in particular, rely on the quality and quantity of the DNA in question. In this study, from 26 individuals ranging in 1-800 years old, the aDNA provided enough quality and quantity that 23 of these samples produced full 24-SNP HIrisPlex profiles for DNA-based eye and hair colour prediction. It is important to take into account however, that although this chapter shows the high success rate and robustness of the HIrisPlex genotyping assay, these results do not allow the conclusion that the HirisPlex assay will always be successful in other old and ancient DNA samples. This is because the success of DNA genotyping with any system or assay strongly depends on the DNA preservation status as well as the amplifiable DNA quantities available. In any case, the results I presented in chapter 4.2, together with those presented in chapter 3, provide evidence that the HIrisPlex assay is sensitive and robust for successful DNA-based eye and hair colour phenotyping from samples of various quality and quantity ranges. This yields great promise for the future of the HIrisPlex system as an application to provide answers to questions from a forensic to historical, anthropological and even evolutionary perspective.

Competing methods that challenge the IrisPlex system for categorical prediction

Since the publication of the IrisPlex system as a whole, there have been discussions on ways to improve the predictability of eye colour from DNA using alternative DNA markers and interpretation methods, especially concerning the intermediate eye colour category. Several groups have challenged its effectiveness in the type of modelling approach used and

its prediction accuracy, suggesting a better approach and the inclusion of different SNPs. There have been several different statistical approaches proposed and published over the last few years for the prediction of phenotypic traits from genetic data. Here we take an in-depth look at the methods used giving examples of eye and hair colour prediction and how they perform on their published data.

Mengel-From *et al.* [27], took a likelihood ratio (LR) approach to prediction. Using 4 SNPs, rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs1129038 (*HERC2*) and rs11636232 (*HERC2*), they reach an LR of 29.3 in discriminating between light and dark eye colour in Europeans [27]. Spichenok *et al.* [28] use a scenario-based exclusion approach with six SNPs for eye colour prediction, rs12913832 (*HERC2*), rs16891982 (*SLC45A2*), rs12203592 (*IRF4*), rs1545397 (*OCA2*), rs6119471 (*ASIP*), and rs885479 (*MC1R*). After genotyping the most highly associated eye colour SNP rs12913832 to indicate either a proposed blue or non-blue eye colour, there is a step-wise inclusion of SNP data from the rest of the set to come to a final conclusion, however there is no measurement of its accuracy or explanation of variance other than the list of samples the scenario-based approach was performed on [28, 29]. As previously mentioned Valenzuela *et al.* [22] found that a combination of three SNPs (rs12913832 (*HERC2*), rs16891982 (*SLC45A2*) and rs1426654 (*SLC24A5*)) were able to explain 76% of eye colour variation and also gave the best prediction for light and dark hair colour, explaining 76% of total hair melanin using a multi-linear regression modelling approach. Sulem *et al.* [3] developed a hair prediction tool based on their own GWA data that was able to exclude red and either blond or brown hair colour in prediction of individuals from its dataset.

More recently, Pospiech *et al.* [30] proposed a Bayesian network model approach using the six highest ranked SNPs from six genes in the Liu *et al.* [12] study. This LR-type approach uses two models, the first model assumes eye colour categories of blue, brown, green and hazel and the second, light and dark. Although they could not improve Liu *et al.* [12] results using this method, they do reach an LR of 32 in discriminating light versus dark eye colour using this SNP set, which is comparable to other studies. As described in their paper [30], the Bayesian approach is a weight of evidence method that is performed quite often in forensic casework. Using a database of 638 individuals it was used to evaluate the frequency of the SNPs for each genotype for each defined eye colour category. Their paper describes the process of Bayesian networks and the results they obtained on their test set of 80 samples. The method is sound by their use of a training and separate test set. It may also be used to help users grasp the idea of trait prediction as they may be familiar with using likelihood ratios, however, it does not improve prediction by any means using this 6 SNP set. Although this study shows that it is possible to use Bayesian networks for trait prediction, the question is, do we really need such a Bayesian network of likelihoods, when a measured prediction probability can be attained from using the IrisPlex approach. STR profiling and the use of LR's have been fully established in the forensic community. Needless to say, it is easier to grasp LR approaches due to this, however looking at the IrisPlex method and its prediction probabilities; it does provide a similar if not better prediction outcome. In my opinion, as DNA phenotyping is merely used as 'intelligence' for an investigation, and eye colour categorical phenotyping is group-based prediction (as we cannot predict individual eye colour yet), then why the need for the production of an LR?

Surely the description of a result being 3 or 4 times more likely to be blue, does not give the most information to the leading police investigator. We come back to the fallacy of what is the definition of 'more likely'? It is better to give real measurements and IrisPlex prediction probabilities gives the probable amount of each eye colour category belonged to the individual. Also with the vigorous testing of the models accuracy parameters in **Chapter 2.3**, every prediction probability, in the case of eye colour the highest category probability is the predicted colour phenotype, has been assessed and a police investigator not only receives the final colour prediction, each category's probability values (which shows what other colour category was close) but they also receive the accuracy at which this result was seen in a population set of > 3800 individuals from several different countries with variable eye colour frequencies. This is not the case for LR's as these values are a simple measurement of ratios.

Following on with Bayesian approaches, most recently Ruiz *et al.* [31] proposed a similar Bayesian likelihood-based classification approach using their *snipper* tool. 256 subjects were used to train their Bayesian model using 3 categories, blue, green-hazel, and brown. Overall, the aim of the study was to show that this LR-based approach including 7 additional SNPs improved eye colour prediction, in particular the intermediate category. It is interesting to see their approach of taking the highest 2 category LR's produced from allelic data combined with their *snipper* tool to assess the ratio for a final prediction (using a threshold of 3:1), this is an improvement to the simple highest LR approach by Pospiech *et al.* [30]. Their own analysis on the 6 core SNPs from Liu *et al.* [12] did not show an improvement in prediction, and only a slight increase is seen with the addition of the other SNPs from the Mengel-from *et al.*

study [27]. However, the largest increase in prediction was observed by the addition of several other SNPs with rs1129038, rs1667394, and rs7183877 from *HERC2* displaying the largest improvement in prediction. In fact, Eriksson *et al.* [32] previously identified both rs1667394 and rs7183877 as being independently significant for eye colour prediction of intermediates. Overall this information is useful to assess as Ruiz *et al.* [31] showed an increase in intermediate predictability with the addition of these markers. It would be useful to further investigate these 2 *HERC2* SNPs and to see how they improve the intermediate category's prediction, perhaps even implementing them into the IrisPlex tool if validated. Also further testing of using rs1129038, a SNP in high LD with rs12913832 should be performed in its use of categorical prediction, we know it shows significant contributions towards quantitative prediction as previously published [21] and **Chapter 3.4** of this thesis. The comparison of AUC values from the method by Ruiz *et al.* [31] versus the IrisPlex system performed in this paper is not to be trusted however, for several reasons. Firstly the sample number is extremely small and therefore the large variation that is known to occur with eye colour and the genotype combinations associated with its variation are unlikely to be represented in this set, including the extra processing of phenotypes through the structure program that ensured 3 separate category entities. Secondly, and more importantly it is statistically incorrect to test a model or method on its training set. It is vital to have a separate test set that was not involved in modelling to accurately assess its performance; therefore its apparent large improvement in eye colour prediction is questionable.

Finally, a more recent publication proposed the use of sex as a prediction variant for eye colour [33] and when included in a prediction

model such as IrisPlex, it could yield more accurate eye colour prediction results overall. It is well known that confounding factors play a part in genetic association, for example some of these factors have been noted previously with quantitative eye colour such as sex and age [14]. However sex was not considered to add significantly to prediction accuracy (it was ranked 16 (Hue) and 14 (Saturation) on the list). Therefore for quantitative eye colour, sex was already known to have a significant effect, however its impact on prediction was very low. Secondly and more importantly, this papers statement that the sex effect can improve categorical prediction was not actually measured using the 6 IrisPlex SNPs, but only the number of correctly predicted females versus males was examined. Therefore without having quantively measured the sex effect for categorical eye colour, it is unclear if it is the sole explanation of a female versus male difference or simply that other factors are impacting on the result, such as population-specific eye colour distribution effects for example. It would therefore be necessary to specifically test this sex effect and quantify its impact on prediction, and rank its effect amongst other variants for the best predictors before concluding that "gender is a major factor explaining discrepancies in eye colour prediction" and assailing the IrisPlex model. If its effect is very minor, then there may be no additional benefit of adding it to a prediction model, as observed in Liu et al {ref} for quantitative eye colour.

To summarise, alternative approaches may be valid, but it is important to compare methods accurately. Perhaps both methods described here can be used, IrisPlex modelling and Bayesian approaches, however further investigation into other SNPs and genes that are responsible for eye colour must first be undertaken. I believe this will be

an on-going debate, as both methods have negative and positive aspects; however it is important to note that both perform the task at hand, which is the prediction of eye colour for 'intelligence' purposes. It is not until the day that we find all the responsible SNPs and genes for eye colour that we can achieve perfect categorical prediction, until then quantitative phenotyping and prediction hold the key.

Future perspectives to overcome the hurdles currently encountered with eye and hair colour prediction

With the establishment and assessment of a tool in both **Chapters 2 and 3**, one recognises their triumphs and misgivings. In this sense, it is simply not the performance of the model, the assay or the system as a whole, but that further work is needed to increase the level of detail at which DNA-based phenotyping can be achieved. In terms of eye colour, this statement is in relation to the intermediate eye colours such as green. Although previous genetic studies on human eye colour are based on categorical trait information, most often a three category scale of blue, green or intermediate, and brown eye colour [3, 4, 6, 8], it is known that in reality iris colour exists on a more continuous spectrum from the lightest shades of blue to the darkest of brown or black [15]. The use of categorised information from continuous traits can oversimplify the true quantitative nature of the trait. This may also explain why it is difficult to predict green or intermediate eye colour where the current AUC prediction from Liu *et al*. [12] is at a lower value of 0.73. Perhaps the SNPs or genes have not yet been recovered from GWAS and other studies due to the previous categorical search approach, as it was found that additional genes contributing to human iris coloration were identifiable if

the full quantitative spectrum of eye coloration was used [21]. Several new genes, *LYST* and *DSCR9*, previously unidentified using the simpler categorical approach, where found when an alternative quantitative approach utilizing a different colour spectrum of Hue (H) and Saturation (S) were applied as phenotype input data to the GWA study. It seems that moving from categorical eye colour prediction towards DNA prediction of various eye colour shades using a more quantitative approach seems to the be the best course of action. This could lead to a better understanding of the processes and genes involved in green/intermediate eye colour association and prediction. Epistatic interactions in particular have also been proposed in both categorical [30, 31] and quantitative [21] methods to improve prediction and a further look at these contributions for these intermediate categories may lead to fruitful results when combined with new independent SNP effects. Therefore the future for eye colour genetics is clear, using a quantitative finer phenotyping method as similar to the approach proposed in **Chapter 2.4** should increase the effectiveness of future GWAS scans. By redefining the term intermediate or green colour into an amount and type of melanin across the iris, this should allow researchers to find the genes and SNPs responsible for the intermediary eye colours produced. Epistatic interactions that have simply not been found yet due to lack of significant signals may also be found using such tactics. Finally taking a more quantitative approach as opposed to a categorical approach may improve prediction with the inclusion of more intermediate-colour associated markers. Following this thesis, one future study should be to apply the new quantitative phenotyping method described in Chapter 2.4 to a new GWAS study on continuous eye colour to try to improve knowledge of the intermediate/green eye colour that is

observed in many individuals. With such a study, it is hoped that new genes and predictive DNA markers will be found that help explain the more subtle variation of human eye colour. If identified, these SNPs shall than be included into DNA-based prediction systems to allow eye colour prediction on a much more detailed level in comparison to the current systems such as IrisPlex and HIrisPlex. I envision a colour chart or colour print-out of the individual's eye colour phenotype as an outcome of the DNA phenotyping prediction, in contrast to a simple category descriptive term as available today. Such a detailed outcome would also avoid uncertainties in the perception of eye colour categories by investigating police officers.

When considering hair colour prediction, there are unfortunately more hurdles to overcome due to the more variable nature of the trait in comparison to eye colour. In addition, with hair colour we face issues such as hair dying that is usually not a problem with eye colour (although coloured contact lenses are available, they are much less widespread than dyed hair). Clearly, this is not something that can be predicted from DNA, however we must think logically about how much this does affect DNA-based hair colour prediction. Firstly, in terms of a crime scene sample prediction, it is generally known that men tend to commit crime more than woman and men tend to dye their hair colour less than women. These are two generalisations, however when combined it does lean in hair colour predictions favour. It is important to acknowledge that there may be circumstances where the person has dyed their hair colour therefore an incorrect prediction may be made. However in general, the main objective of hair dying in adult men is to hide the progression of greying, therefore men tend to keep to their natural hair colour category,

it is less frequent to find men who would change their hair colour category entirely, i.e. go from black hair to blond hair. However, these circumstances do exist, and they do dampen hair colour prediction findings, therefore it is necessary to make individuals that use HIrisPlex aware of this issue. Secondly environmental conditions also affect hair colour. The changes that are observed in regions with increased sun exposure tend to lead to lighter colour shades. Yet again, although it is a factor in hair colour prediction, it is unlikely that the sun has the capability of changing an individual's entire category, i.e. black to blond, however it may be more difficult and a cause for concern with the blond/dark blond (light brown) predictions, and this should also be made aware to the user.

Recent publications have been done in the last few years that have tried to pinpoint the genes and processes that enable the loss of hair colour pigment that lead to the white or grey lack of colour in hair follicles of older adults, although not to the extent at which they have been performed on associations with full colour categories. The mechanism of action [34] in hair greying or whitening is quite different to other hair colour changes that occur from a child to adult in certain individuals, therefore more analyses need to be performed. Research over the last few years into the genes associated with hair colour greying have shown that it is linked with the self-maintenance of melanocyte stem cells, and greying is accelerated by bcl-2 deficiency performed on mouse-models [35], perhaps future human studies can provide us with the genes and SNPs necessary to be included in the set of HIrisPlex hair colour predictive DNA variants to better the prediction system and include grey hair as a type of category.

The utility of the HIrisPlex system is the prediction of adult hair

colour before the onset of greying. The natural change in hair colour over one's lifetime before loss of colour i.e., greying starts, is also another hurdle for hair colour prediction however it is one that further research may help leap over. The second most obvious age-dependent hair colour change that occurs in certain individuals is the change from light blond during childhood towards dark blond/light brown as an adult, but can also occur in rare circumstances from light brown to dark brown/almost black. Suggestions of hormonal changes during adolescence have been advocated as a possible explanation [36], but the molecular basis is still not yet known. These age dependent blond to brown hair colour changes and the effect they have on prediction came to obvious light using the HIrisPlex prediction system in **Chapter 3.1.** Using the HIrisPlex system for hair colour prediction, we noticed an obvious trend in individuals that were incorrectly predicted with a high probability of light blond, when their actual phenotype was light to dark brown, to have had a different hair colour as a child (i.e. blond). It appears that the hair colour of these individuals changed over the years to darker colours (brown), however using some of the highest-ranking variants in hair colour association (HIrisPlex), why does the prediction give their childhood hair colour and not their adult hair colour in these circumstances. We found a high and statistically significant correlation (Pearson correlation $r^2 = 0.81$, $p < 0.01$) between the increase in brown (darkening of hair) and the increase in age since the hair colour change occurred for the few Irish individuals with whom we had hair imagery, phenotype and genotype information for this phenomenon. This is certainly an intriguing outcome, and has begged the question as to what underlying molecular processes are involved in age-dependent non-grey hair colour changes which should be a focus in

future research. It may indeed be expected that there is biological control of age-dependent hair colour changes. But what is it? One obvious candidate would be gene regulation and perhaps future studies into DNA methylation may reveal an answer.

There are many more unanswered questions in hair colour prediction than eye colour prediction due to the variable nature of the trait and the more obvious intrinsic changes that occur in one's lifetime. This occurs more often with hair colour than eye colour, apart from the noted loss of colour intensity towards blue or lack of pigment [37] as a person ages, that is quite similar to hair greying. Also, there are some babies born with blue eyes that change to brown eyes during the first weeks after birth. However, this effect is more than likely a slight delay in the expression of pigmentation genes as indicated by the fact that gene mapping and prediction studies done in adults were more successful to explain and predict eye colour over hair colour. However, future research remains the key in overcoming the limitations we see today in DNA phenotyping of eye and hair colour. Quantitative phenotyping and prediction for hair colour is also expected to be more difficult than I have shown for eye colour, as individuals have the ability to produce every hair colour category in each hair follicle, while displaying one overall colour. However, in principle it is possible to use quantitative measurements to find more genes associated with quantitative measures of hair colour. Studies by Mengel-From *et al.* [38] and Velenzuela *et al.* [22] revealed additional genes that may not have been found via the simple categorical approach such as *SLC24A5*, *MYO5A*. *MYO7A*, *MLPH, GPR143, DCT, HPS3, GNAS, PRKARIA, ERCC6*, and *DTNBP1*. It shall be possible to adapt the new method from **Chapter 2.4** for hair colour phenotyping provided that

high-quality hair images are used. Quantitative fine phenotyping coupled with gene mapping and particularly functional studies are the key to understanding the genetic basis and predictability of eye and more so hair colour. This may in turn also help answer what genes are affected in the age-dependent hair colour changes, including hair colour loss over one's life time.

Having discussed eye and hair colour genetics extensively in this thesis but at the same time knowing the phenotypic, biological and more specifically genetic relationship between all three human pigmentation traits, may trigger the reader to ask: But what about skin colour? At present, skin colour phenotyping is not at the level of eye and hair colour DNA-based prediction and only a handful of studies have attempted skin colour prediction i.e Valenzuela *et al.* [22]. That particular study showed that the amount of skin colour variance that can be explained by known DNA markers is only half of the variance levels that can explain eye colour using DNA. This illustrates that currently known pigmentation SNPs are not enough to perform DNA-based skin colour prediction in practical applications. One reason for the lack of knowledge on skin colour genes, relative to eye and hair colour genes is that in contrast to European eye and hair colour variation, skin colour varies mostly between populations from different continental regions such as Asia, Africa and Europe. Such a globally dispersed complex trait poses major challenges to gene mapping studies, at least using the commonly applied genome-wide association approach. To obtain the maximum degree of phenotypic variation for a skin colour GWAS, one would have to pool Europeans, Africans and Asians in the data analysis. However, this approach would not only highlight true skin colour genes but also many other genes that show differences

between continental human populations without any involvement in skin colour. Furthermore, it would be impossible to separate the true positive from the false positive findings in such a study. Therefore, GWAS studies on skin colour can only be done within continental groups such as Europeans [3, 6], or within Asians [39], but because there is only limited skin colour phenotypic variation within such continental groups, the gene lists identified are largely incomplete. More alternative approaches to GWAS will need to be performed to identify the full mask of skin colour genes before practically useful DNA-based skin colour prediction can be achievable. For now, the predictive capabilities of currently known pigmentation SNPs as well as an understanding of the genetic variation underlying skin colour within continental groups needs to be performed. Perhaps these studies could be enhanced by more detailed skin colour phenotyping, such as using an adapted quantitative approach as used in chapter 2.4 for eye colour. This would be in contrast to the categorical measures of skin colour such as skin types as applied thus far in gene mapping studies [40]. However, in time and using the appropriate methods for identifying more skin colour genes and thus more skin colour predictive DNA variants, it can be expected that skin colour will be combinable with already available traits such as eye and hair colour. This would provide the complete physical appearance picture of human pigmentation traits in DNA phenotyping for various applications.

Concluding remarks

The work shown in this thesis represents the start of DNA phenotyping as an entity. It provides solid practical applications in the use of eye and hair

colour prediction, and expands knowledge on the usefulness of DNA phenoyping in several disciplines. It also highlights new avenues of research to be pursued in the future. This thesis brings together the categorical prediction of two of the most obvious EVCs, eye and hair colour and the first developmentally validated systems created for their prediction from DNA, IrisPlex and HIrsiPlex, for immediate use in forensic and other laboratories worldwide. As previously mentioned, the immediate future of eye and hair colour lies in quantitative prediction and that should be the next course of action to further develop eye and hair colour associated and predictive DNA variants through finer phenotyping in GWA studies, candidate gene or sequencing approaches. By demonstrating with scientific knowledge, and by providing practically applicable laboratory and statistical tools that DNA-based eye and hair colour prediction is possible, my work provides important evidence that forensic DNA phenotyping is indeed a feasible concept. It also shows that DNA phenotyping involving appearance traits has relevance in the anthropological and evolutionary sciences. In the future, eye and hair colour prediction may eventually be combined with not only skin colour [41] but also with chronological age, estimated as an age group [42], and perhaps body height, at least when it comes to height extremes [43]. At present, appearance traits can be predicted from DNA representing group-specific but not individual-specific traits, but it is hoped that one day individual-based appearance prediction will soon become a reality. The day this occurs will mark DNA phenotyping as the ultimate eye-witness tool.

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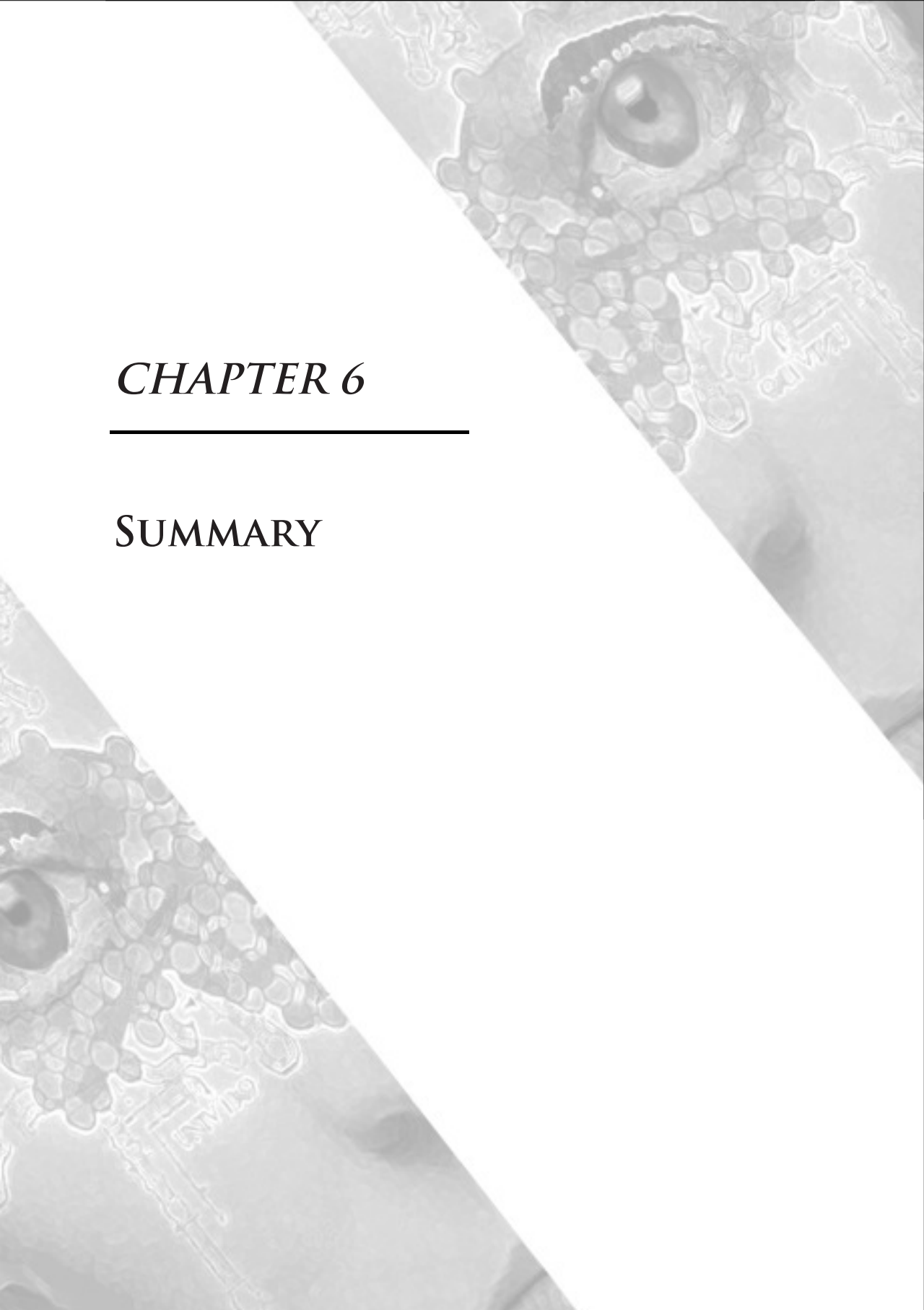


CHAPTER 6

Summary

Phenotyping is the ability to assign characteristics to an organism based on certain measurable parameters. In the case of DNA phenotyping, it is limited to the sole use of genetic information such as DNA to determine a phenotype. DNA phenotyping of externally visible traits is of relevance in a forensic and non-forensic environment. In a forensic setting, it encompasses the ability of a forensic investigator to predict the physical appearance of an individual using molecular analyses from biological samples left at a crime scene. This information is termed DNA 'intelligence'. It is able to provide investigative leads to find unknown perpetrators or identify missing persons in cases where all other means of inquiry, including conventional DNA profiling, are non-informative. In a non-forensic setting, it permits the prediction of the physical appearance of our ancestors, historical persons or any other deceased individual for whom the identification of appearance traits may be interesting, and it sheds light on human evolution. In this thesis, DNA variants highly associated with human eye and hair colour pigmentation traits were explored for their potential and contribution towards accurate prediction systems using sensitive laboratory assays together with statistical models. The DNA-based prediction systems produced were then applied in the forensic, evolutionary, anthropological and historical context.

In **Chapter 1,** I begin with an introduction to the relevance of DNA phenotyping and the biology of human pigmentation traits. This is followed by a brief summary on knowledge from human pigmentation genetics available at the time this thesis work began.

In **Chapter 2,** I focus on DNA-based eye colour prediction. In **Chapter 2.1,** I describe the design, development and performance of the new IrisPlex system, which represents a sensitive single multiplex

genotying assay together with a statistical model based on a large database of genotype and phenotype data. IrisPlex is capable of predicting eye colour using 6 SNPs from 6 genes with high degrees of blue and brown eye colour accuracy. Exploring worldwide populations using this system allowed the conclusion that IrisPlex-based eye colour prediction is reliable independent of bio-geographic ancestry knowledge. In **Chapter 2.2**. I present a critical assessement of the IrisPlex genotyping assay by following internationally accepted guidelines. The assay underwent vigorous tests on species specificity, sensitivity, stability, mixture studies, reproducibility, precision and accuracy, casework samples and population studies for it to be developmentally validated for use in forensic casework in any accredited laboratory. I concluded that the IrisPlex assay is a highly sensitive system that is successfully able to cope with the specific requirements of forensic DNA analysis. In **Chapter 2.3**, I present the evaluation of the IrisPlex model on over 3800 individuals from seven sites across Europe which revealed that blue and brown eye colour can be predicted with accuracies > 94%. Upon critical assessment of the results, I found the best threshold for a balance between model sensitivity and specificity to be a prediction probability value > 0.7. In **Chapter 2.4**, I describe a new method of quantitative eye colour phenotyping that estimates eumelanin amount, pheomelanin amount and lack of pigment in an iris from high-resolution digital imagery. This new approach allows a better separation of irises according to melanin amount rather than previous methods of colour. Using the currently known most informative eye colour SNPs, this new method produced improved measurements that explain the variance observed in eye colour (eumelanin at 57%, pheomelanin at 44% and no pigment at 74%). This method also

highlighted several epistatic interactions between SNPs that remained unidentified with previously used methods. Hence, this method if applied to future genome-wide association studies has great potential to highlight new eye colour genes and identify additional eye colour predictive DNA markers.

In **Chapter 3,** I focus on DNA-based hair colour prediction. In **Chapter 3.1**, I describe the design, development and performance of the new HIrisPlex system, which is made up of a sensitive single multiplex genotyping assay together with two prediction models, one for eye colour and the other for hair colour. Targeting 24 of the most eye and/or hair colour informative DNA variants, this system has the ability to simultaneously predict both the eye and hair colour of an individual from a DNA sample. HIrisPlex displays accuracies of > 94% for eye colour category prediction as seen in **Chapter 2.1**, together with accuracies of 79% on average for hair colour categories and is reliable independent of ancestry knowledge. In **Chapter 3.2**, I show how the HIrisPlex assay underwent vigorous testing quite like those seen in **Chapter 2.2** in the IrisPlex assay validation. The HIrisPlex assay is highly sensitive and passed all the required international forensic guidelines for use worldwide.

In **Chapter 4,** I show how the IrisPlex and HIrisPlex eye and hair colour prediction systems can be applied in a non-forensic environment such as biological anthropology. **Chapter 4.1** uses knowledge from HIrisPlex data across modern day Europe and the world to reveal the eye and hair colour predictions of ancient samples from published data evidence, such as the Neanderthal, Denisova and the Tyrolean Iceman. The Iceman was predicted to have brown eyes and dark brown hair. However, several derived alleles associated with light pigmentation in

modern Europeans were found in his profile that suggest eye and hair colour variation arose well before he existed, over 5,300 years ago. Newly acquired aDNA samples (4,950-4,850 cal BP) also lend support to this theory, revealing the presence of the *HERC2* rs12913832 G allele, which is highly indicative for derived light eye and hair colour at this time period. The chapter concludes with propositions that eye and hair colour variation arose well before 5,300 years ago, the first data-supported timestamp to exist. **Chapter 4.2** focuses on the analysis of 'younger' aDNA samples (up to 800 years old) that utilise the whole HIrisPlex system. aDNA was extracted from bone and teeth samples and the sensitive HIrisPlex assay provided full 24-SNP profiles in 23 out of the 26 aDNA samples tested. From this data, a historical figure from Polish history, General Sikorski was revealed to have blue eyes and blond hair, confirming historical records. These two chapters not only demonstrate interesting applications of eye and hair colour DNA phenotyping in the evolutionary, anthropological and historical context, but also illustrate that the HIrisPlex assay is highly robust and sensitive enough to allow eye and hair colour phenotyping from ancient DNA.

Finally, in **Chapter 5**, the results are discussed in a more general manner, and the findings are ordered in a broader context so that readers may fully understand the scope of the thesis, the overall achievements and their relevance. In addition, current challenges in DNA-based human pigmentation prediction are addressed and an outlook at future perspectives in DNA phenotyping of human physical appearance is presented.

SAMENVATTING

Met fenotyperen is het mogelijk om eigenschappen toe te kennen aan een organisme gebaseerd op bepaalde meetbare parameters. In het geval van DNA fenotyperen, het bepalen van een fenotype is gelimiteerd door enkel het gebruik van genetisch informatie zoals DNA. DNA fenotyperen van uiterlijke zichtbare kenmerken bestaat in zowel forensische als niet-forensische kringen. In een forensische setting omvat het de mogelijkheid voor een forensische onderzoeker om de fysieke verschijning van een individu te voorspellen aan de hand van moleculaire analyses op biologische monsters die zijn aangetroffen op een plaats delict. Dit soort informatie wordt DNA 'intelligence' genoemd. Het is hiermee mogelijk om onderzoekslijnen te verschaffen die kunnen leiden tot het vinden van onbekende daders of tot het identificeren van vermiste personen in zaken waarin alle andere onderzoeksmiddelen, inclusief conventioneel DNA profilering niet de nodige informatie opleveren. In een niet-forensische setting is het mogelijk om de fysieke verschijning te voorspellen van onze voorouders, historische personen of elk andere overleden individu van wie de identificatie van diens uiterlijke kenmerken interessant zou kunnen zijn, en dit werpt een licht op de evolutie van de mens. In dit proefschrift zijn DNA varianten onderzocht die zowel sterk geassocieerd zijn met, alsmede een sterk voorspellende waarde hebben voor de oog en haarkleur van de mens. Er is specifiek onderzocht wat hun potentie en contributie is ten opzichte van een nauwkeurig voorspellingssysteem, gebruikmakend van gevoelige laboratorium opstellingen in combinatie met statistische modellen. De geproduceerde DNA-gebaseerde voorspellingssystemen zijn vervolgens toegepast in de forensische, evolutionaire, antropologische en historische context.

In **Hoofdstuk 1** start ik met een introductie over de relevantie van DNA fenotyperen en de biologie van de pigmentatie eigenschappen van de mens. Dit wordt gevolgd door een korte samenvatting van de genetica van de humane pigmentatie, gebaseerd op de beschikbare kennis ten tijde van het opschrijven van dit proefschrift.

In **Hoofdstuk 2** focus ik op DNA-gebaseerde oogkleur voorspelling. In **Hoofdstuk 2.1** beschrijf ik het ontwerp, de ontwikkeling en de prestaties van het nieuwe IrisPlex systeem; een gevoelige en enkelvoudige multiplex test in combinatie met een statistisch model dat is gebaseerd op een grote database met genotype en fenotype informatie. Irisplex is in staat om oogkleur te voorspellen, gebruikmakend van 6 SNPs uit 6 genen, met een hoge graad van populatie-gebaseerde nauwkeurigheid. Met dit systeem zijn populaties van over de hele wereld onderzocht, wat leidde tot de conclusie dat oogkleur voorspelling met het IrisPlex systeem betrouwbaar is, onafhankelijk van kennis over de bio-geografische afkomst. In **Hoofdstuk 2.2** presenteer ik een kritische toetsing van de IrisPlex genotyperingstest door middel van het volgen van internationaal geaccepteerde richtlijnen. De test is intensief getoetst op soort-specificiteit, sensitiviteit, stabiliteit en mix studies, reproduceerbaarheid, precisie en nauwkeurigheid, casework monsters en populatie studies, voordat het kon worden ontwikkeld en gevalideerd voor gebruik in forensische onderzoek van zaken in elk geaccrediteerd laboratorium. Ik heb kunnen concluderen dat de IrisPlex test een erg gevoelig systeem is dat op succesvolle wijze tegemoet kan komen aan de specifieke eisen van forensische DNA analyse. In **Hoofdstuk 2.3** presenteer ik de evaluatie van het IrisPlex model in meer dan 3800 individuen afkomstig vanuit 7 plaatsen verspreid over Europa. Hieruit

bleek dat blauwe en bruine oogkleur kunnen worden voorspeld met een nauwkeurigheid van > 94%. Na kritische toetsing van de resultaten, heb ik de beste drempelwaarde vastgesteld voor een balans tussen model sensitiviteit en specificiteit op een voorspellingswaarschijnlijkheid van > 0.7. In **Hoofdstuk 2.4** beschrijf ik een nieuwe methode voor de kwantitatieve oogkleur fenotypering dat de hoeveelheid eumelanine, pheomelanine en gebrek aan pigment in een iris inschat aan de hand van hoge-resolutie digitale beelden. Deze nieuwe aanpak maakt het mogelijk om irissen op een betere wijze van elkaar te onderscheiden gebruikmakend van de ingeschatte melanine hoeveelheid in plaats van voorgaande methodes die gebaseerd zijn op de waargenomen kleur. Door de SNPs te gebruiken die momenteel bekend zijn als beste oogkleur kwantitatief te kunnen voorspellen (n=12), heeft deze vernieuwde methode verbeterde metingen geproduceerd die de variatie in oogkleur (eumelanin op 57%, pheomelanine op 44% en geen pigment op 74%) kunnen verklaren. Deze methode heeft ook verschillende epistatische interacties tussen SNPs aangetoond, die niet geïdentificeerd werden met voorgaande methodes. En daarom zou deze methode, indien toegepast in toekomstige genome-brede associatie studies een groot potentieel hebben om nieuwe oogkleur genen aan te tonen en om extra oogkleur-voorspellende DNA markers te identificeren.

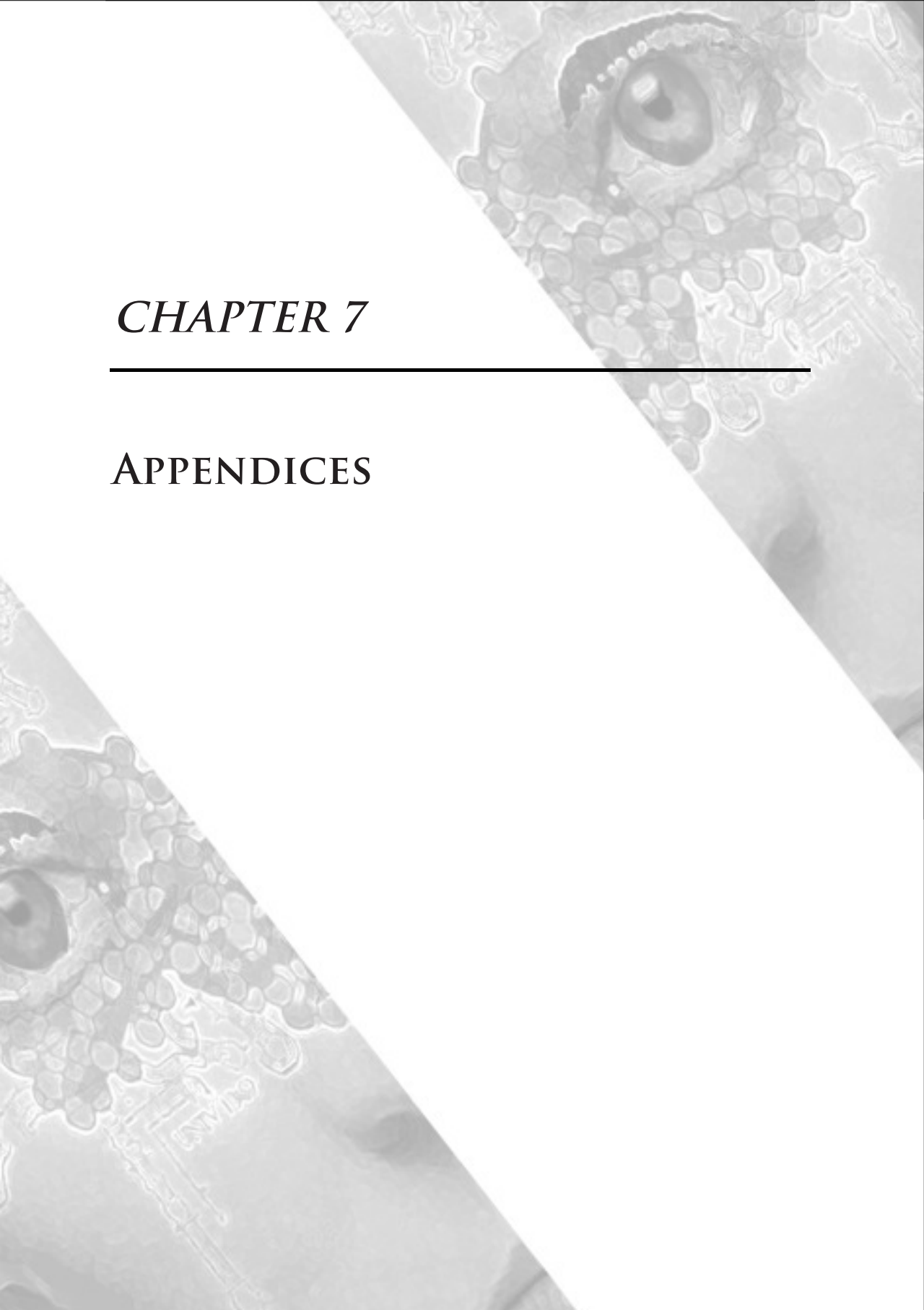
In **Hoofdstuk 3** focus ik op DNA-gebaseerde haarkleur voorspelling. In **Hoofdstuk 3.1** beschrijf ik het ontwerp, de ontwikkeling en de prestaties van het nieuwe HIrisPlex systeem, welke is opgebouwd uit een sensitieve, enkelvoudige multiplex test in combinatie met twee nauwkeurige voorspellingsmodellen, één voor oogkleur en de andere voor haarkleur en schakering. Dit systeem maakt gebruik van 24 DNA

varianten die het meest informatief zijn voor oog en/of haarkleur, het is in staat om vanuit een spoor gevonden op een plaats delict, tegelijkertijd de oog en de haarkleur van een individu te voorspellen. HIrisPlex laat nauwkeurigheden zien van > 94% voor oogkleur categorische voorspelling, zoals is beschreven in **Hoofdstuk 2.1**, in combinatie met nauwkeurigheden van > 79% gemiddeld genomen voor haarkleur categorieën, en is betrouwbaar ongeacht de geografische afkomst van de donor van het monster. In **Hoofdstuk 3.2** laat ik zien hoe het HIrisPlex systeem intensieve testen is ondergaan, zoals is beschreven in **Hoofdstuk 2.2** voor de validatie van de IrisPlex test. De HIrisPlex test is zeer gevoelig en heeft voldaan aan alle vereiste internationale forensische richtlijnen voor wereldwijd gebruik.

In **Hoofdstuk 4** laat ik zien hoe de IrisPlex en HIrisPlex oog- en haarkleur voorspellingssystemen kunnen worden toegepast in een niet-forensische werkveld zoals biologische antropologie. **Hoofdstuk 4.1** beschrijft hoe kennis uit de HIrisPlex data van het moderne Europa en de wereld, gebruikt wordt om de oog en haarkleur te voorspellen van bijvoorbeeld de Neanderthaler, Denisova en de Tyroolse Ijsman. Hiervoor werd gebruik gemaakt van reeds gepubliceerde DNA-sequentie data. Voor de Ijsman werden bruine ogen en donkerbruin haar voorspeld. Echter, in zijn profiel zijn ook verschillende afgeleide allelen gevonden die geassocieerd zijn met lichte pigmentatie in moderne Europeanen, dit suggereert dat oog en haarkleur variatie al was ontstaan lang voor het bestaan van de Ijsman, meer dan 5300 jaar geleden. Deze theorie werd ondersteund door analyses van nieuw verkregen aDNA monsters (4950-4850 jaar voor nu), deze lieten zien dat het *HERC2* rs12913832 G allel, welke zeer indicatief is voor afgeleide lichte oog en haarkleur, aanwezig

was in deze periode. Dit hoofdstuk wordt afgesloten met de propositie dat oog en haarkleur variatie ontstond ruim voor het jaar 5300, tot op heden het eerste tijdspunt dat door data wordt ondersteund. **Hoofdstuk 4.2** richt zich op de analyse van relatief gezien 'jonger' aDNA (tot ongeveer 800 jaar oud) waarbij het gehele HIrisPlex systeem wordt gebruikt. aDNA werd geextraheerd uit bot en tand monsters en er werd een volledig 24-SNP profiel verkregen met het HIrisPlex systeem in 23 van de 26 aDNA monsters die getest werden. Uit deze data kwam naar voren dat een historisch figuur uit de Poolse geschiedenis, Generaal Sikorski blauwe ogen en blond haar had, en dit resultaat bevestigde de geschiedenis annalen. Deze twee hoofdstukken laten niet alleen interessante toepassingen zien voor oog en haarkleur DNA fenotyperingen in de evolutionaire, antropologische en historische context, maar het illustreert ook dat het HIrisPlex systeem zeer robuust en sensitief genoeg is om oog en haarkleur te fenotyperen vanuit aDNA.

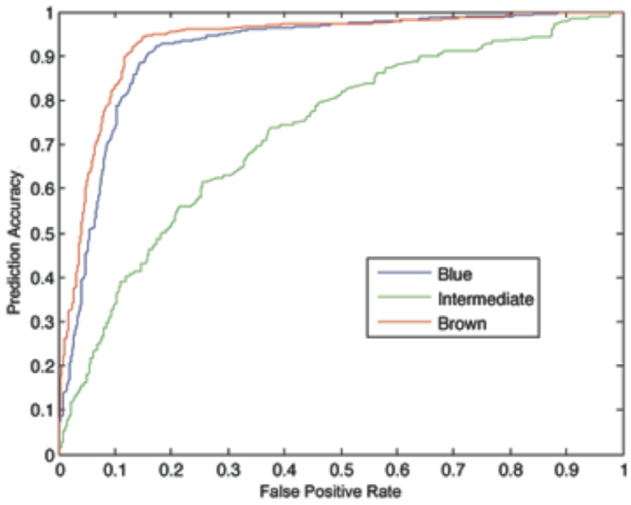
Als laatste, in **Hoofdstuk 5** worden de resultaten bediscussieerd in een meer algemene manier, waarbij de bevindingen zijn geordend in een bredere context, om zodoende het doel van dit proefschrift, de algehele verworven resultaten en hun relevantie aan de lezers volledig te kunnen verduidelijken. Ook zullen huidige uitdagingen in DNA-gebaseerd voorspellingen van menselijke pigmentatie worden geadresseerd en worden toekomstige perspectieven in DNA fenotypering van de humane fysieke verschijning gepresenteerd.



CHAPTER 7

Appendices

IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Science International Genetics 5 (2011) 170-180.



Supplementary Figure 1: ROC curve of Dutch European cohort (n = 2364) prepared from previously published data (Liu et al. Curr. Biol. 19 (2009) R192-R193), brown, blue and green curves represent brown, blue and intermediate eye colour respectively

Image number	Sex	Country of Origin	rs12913832	rs1800407	rs12896399	rs16891982	rs1393350	rs12203592	Brown (p)	Intermediate(p)	Blue (p)
1	F	Netherlands	CC	CC	TT	GG	TT	GG	0,01	0,02	0,97
2	F	New Zealand	CC	CC	TT	GG	CT	GG	0,01	0,03	0,96
3	F	Netherlands	CC	CC	TT	GG	CT	GG	0,01	0,03	0,96
4	M	Netherlands	CC	CC	TT	GG	CC	GA	0,01	0,03	0,96
5	M	Netherlands	CC	CC	TT	GG	CC	GG	0,02	0,03	0,95
6	F	Netherlands	CC	CC	TT	GG	CC	GG	0,02	0,03	0,95
7	F	Netherlands	CC	CC	TT	GG	CC	GG	0,02	0,03	0,95
8	F	Ireland	CC	CC	GT	GG	CT	AA	0,01	0,05	0,94
9	M	Netherlands	CC	CC	GT	GG	CT	GG	0,02	0,04	0,94
10	M	Netherlands	CC	CC	GT	GG	CT	GG	0,02	0,04	0,94
11	F	Netherlands	CC	CC	GT	GG	CT	GG	0,02	0,04	0,94
12	M	Estonia	CC	CC	GT	GG	CT	GG	0,02	0,04	0,94
13	F	Netherlands	CC	CC	GG	GG	CT	GG	0,03	0,07	0,9
14	F	Netherlands	CC	CC	GG	GG	CT	GG	0,03	0,07	0,9
15	F	Netherlands	CC	CC	GG	GG	CT	GG	0,03	0,07	0,9
16	M	Poland	CC	CC	GG	GG	CT	GG	0,03	0,07	0,9
17	M	Netherlands	CC	CC	GG	GG	CT	GG	0,03	0,07	0,9
18	M	Netherlands	CC	CC	GG	GG	CC	GG	0,05	0,08	0,87
19	M	Netherlands	CC	CC	GG	GG	CC	GG	0,05	0,08	0,87
20	M	Germany	CC	CC	GG	GG	CC	GG	0,05	0,08	0,87
21	F	Germany	CC	CC	GG	GG	CC	GG	0,05	0,08	0,87
22	M	Russia	CC	CC	GG	GG	CC	GG	0,05	0,08	0,87
23	M	Ireland	CT	CT	GG	GG	CT	AA	0,17	0,49	0,34
24	M	Netherlands	CT	CC	GG	GG	CT	GG	0,69	0,18	0,13
25	M	Spain	CT	CT	TT	GG	CC	GG	0,36	0,22	0,42
26	F	Netherlands	CT	CT	GT	GG	CC	GG	0,45	0,25	0,3
27	M	Spain	CT	CC	TT	GG	CT	GG	0,55	0,14	0,31
28	M	Netherlands	CT	CC	TT	GG	CT	GG	0,55	0,14	0,31
29	M	Netherlands	CT	CC	TT	GG	CC	GG	0,64	0,13	0,23
30	M	Netherlands	CT	CC	TT	GG	CC	GG	0,64	0,13	0,23
31	M	Netherlands	CT	CC	GG	GG	CT	GG	0,69	0,18	0,13
32	F	Portugal	CT	CC	GG	GG	CC	GG	0,76	0,15	0,09
33	F	Netherlands	CT	CC	GG	GG	CC	GG	0,76	0,15	0,09
34	F	Serbia	TT	CC	GG	GG	CT	GA	0,93	0,06	0,01
35	M	Iran	TT	CC	GG	GG	TT	GG	0,96	0,04	0
36	M	Turkey	TT	CC	GG	GG	CT	GG	0,97	0,03	0
37	F	Suriname	TT	CC	GG	CC	CC	GG	0,97	0,03	0
38	F	Suriname	TT	CC	GG	CC	CC	GG	0,99	0,01	0
39	F	Suriname	TT	CC	GT	CC	CC	GG	0,99	0,01	0
40	F	China	TT	CC	GG	CC	CC	GG	0,99	0,01	0

Supplementary Table 1: Irisplex 6 SNP genotypes and eye colour prediction probabilities together with, sex and country of birth of the 40 individuals used for illustrative purpose

Supplementary Table 2: IrisPlex 6 SNP genotypes and eye color prediction probabilities together with sample details of 934 worldwide individuals from 51 populations of the H952 HGDP-CEPH set (see http://www.sciencedirect.com/science/article/pii/S1872497310000323)

		Blue vs Brown	Intermediate vs Brown	Eye colour associated with minor allele
		α1	α2	
		3,94	0,65	
SNP	Prediction Rank	Minor Allele	β (π1)	β (π2)
rs12913832	1	A	-4,81	-1,79	Brown
rs1800407	2	T	1,40	0,87	Blue
rs12896399	3	G	-0,58	-0,03	Brown
rs16891982	4	C	-1,30	-0,50	Brown
rs1393350	5	A	0,47	0,27	Blue
rs12203592	6	T	0,70	0,73	Blue

Supplementary Table 3: α and β model parameters for 6 SNP eye colour prediction from previously published data (Liu et al. Curr. Biol. 19 (2009) R192-R193)

Supplementary Table 4: Interactive macro for IrisPlex eye colour prediction modelling

(see http://www.sciencedirect.com/science/article/pii/S1872497310000323)

Developmental validation of the IrisPlex system: Determination of blue and brown iris colour for forensic intelligence. Forensic Science International Genetics 5 (2011) 464-471.

Sample	Type	Simulated casework scenario	DNA amount (pg)	Profile quality	Analyst Interpretation
1	Frozen Blood	Intentional heme inhibition	154	All peaks extremely low	Inhibition present
2	Hair	Anagen hair extraction	865	Full profile	single source, blue 0.921p
3	Buccal swab	Single source	1000	Full profile	single source, blue 0.928p
4	Semen	Single source	1000	Low peak heights, full profile	single source, blue 0.937p
5	Touched item Blue+Blue	Trace DNA, mixture	27	Low peak heights, but possible full profile	Probable mixture, due to imbalance at rs1393350
6	Hair	Anagen hair extraction	1000	Full profile	single source blue 0.87p
7	Blood	Single source	1000	Full profile (low peak height at rs 12203592	single source, blue 0.87p
8	Blood	Single source	1000	Full profile	single source 0.94p
9	Hair	Telogen hair extraction	2	Low peak heights, allele dropout	Inadequate DNA quantity
10	Semen	Single source	1000	Full profile	single source blue 0.96p
11	Semen	Single source	1000	Full profile	single source blue 0.96p
12	Touched item Brown+Blue	Intentional mixture	none	No result	Inadequate DNA quantity
13	Blood	Intentional heme inhibition	133	No result	Inhibition present
14	Touched item	Trace DNA, single source	97	Low peak heights, full profile	single source 0.87p blue
15	Negative	Negative control	none	No result	Inadequate DNA quantity
16	Hair	Telogen hair extraction	none	No result	Inadequate DNA quantity
17	Semen	Single source	1000	Full profile	single source blue 0.0.937p
18	Touched item Brown + Blue	Trace DNA, mixture	23	Probable mixture	Probable mixture due to heterozygosity at rs16891982 G/C
19	Blood	Single source	1000	Full profile	single source 0.87p blue
20	Blood	Single source	1000	Low peak heights, full profile	single source 0.95p blue
21	Hair	Catagen hair extraction	1000	Low peak heights, full profile	single source 0.96p blue
22	Semen	Single source	1000	Full profile	single source 0.96p blue
23	Hair	Telogen hair extraction	none	No result	Inadequate DNA quantity
24	Touched item Blue+Blue	Trace DNA, mixture	2	Low peak heights, allele dropout	Inadequate DNA quantity
25	Blood	Single source	1000	Full profile	single source 0.96p blue
26	Semen	Single source	1000	Low peak heights, full profile	single source 0.95p blue
27	Touched item	Trace DNA, single source	266	Full profile	single source 0.552p probable intermediate brown phenotype
28	Touched item Brown + Blue	Trace DNA, mixture	45	Probable mixture	Probable mixture due to heterozygosity at rs16891982 G/C

Supplementary Table 1: Simulated casework samples examined in a blind proficiency test where only concentrations, without sample type or preparation information, were provided to the analyst.

			Blue vs Brown		Intermediate vs Brown
			α1		α2
			3.84		0.37
SNP	Rank	Minor Allele	β (π1)		β (π2)
rs12913832	1	A	-4.87		-1.99
rs1800407	2	T	1.15		1.05
rs12896399	3	G	-0.53		-0.01
rs16891982	4	C	-1.53		-0.74
rs1393350	5	A	0.44		0.26
rs12203592	6	T	0.60		0.69

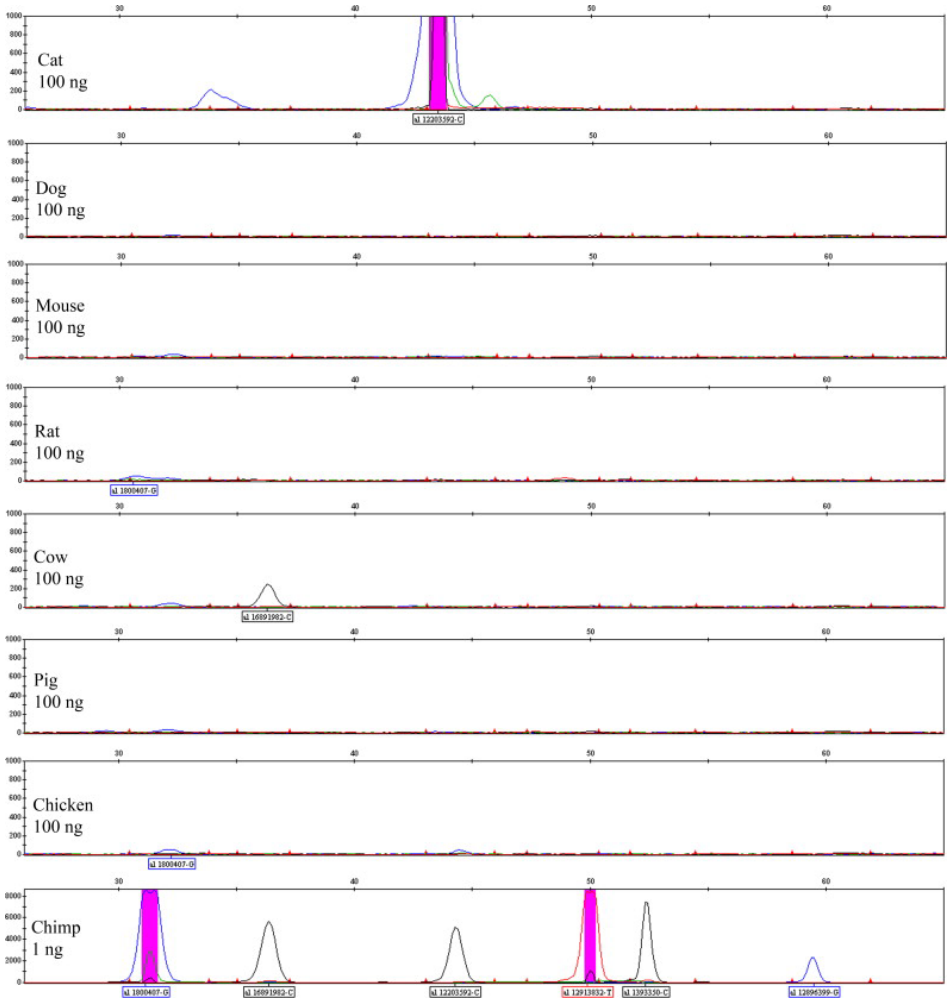
Supplementary Table 2: α and β parameters for the IrisPlex eye colour prediction model

Supplementary Table 3:
Interactive macro for IrisPlex eye colour prediction modelling (Found at http://www.sciencedirect.com/science/article/pii/S1872497310001614)

No.	Sample	DNA input (pg)	External Laboratory 1	External Laboratory 2
1	Ind. 1	192	√	√
2	Ind. 2	191	√	√
3	Ind. 3	213	√	√
4	Ind. 4	219	√	√
5	Ind. 5	180	√	√
6	Ind. 6	265	√	√
7	chimp	78 ng	√	√
8	Ind.7	436	√	√
9	Ind. 8	534	√	√
10	blank	-	√	√
11	blank	-	√	√
12	Ind. 7	124	√	√
13	Ind. 9	281	√	√
14	Ind. 1	192	√	√
15	Ind. 9	58	√	X d/o
16	Ind. 6	265	√	√
17	Ind. 8	299	√	√
18	mix blu1:br1	900	√*	√*
19	Ind. 2	191	√	√
20	blank	-	√	√
21	blank	-	√	√
22	Ind. 7	436	√	√
23	Ind. 9	281	√	√
24	mix blu1:br1	900	√*	√*
25	Ind. 5	180	√	√
26	Ind. 8	534	√	√
27	mix br1:blu4	620	√*	√*
28	Ind. 3	213	√	√
29	Ind. 9	58	√	X d/o
30	mix br1:blu4	600	√*	√*
31	Ind. 7	124	√	√
32	mix blu1:br4	600	√*	√*
33	Ind. 8	299	√	√
34	Ind. 4	219	√	√
35	mix blu1:br4	600	√*	√*
36	chimp	78ng	√	√
37	blank	-	√	√
38	blank	-	√	√
39	Ind. 10	379	√	√
40	Ind. 10	380	√	√

* mixtures are called at user's discretion but in general, heterozygotes were called, even with peak imbalance, once a peak was over 50 RFU.

Supplementary Table 4: The result of concordance testing from two external laboratories. √ indicates a correct genotype and interpretation result was obtained. X indicates an incorrect genotype and drop out (d/o) was called if an allele was missing from the concordance comparison.



Supplementary Figure 1: Human specificity testing of the IrisPlex assay for each SNP on several animal species. The presence or absence of a SNP peak is shown. In the species with 100 ng DNA input, the peak height scale is up to 1000 RFU. The Chimpanzee DNA input is 1 ng with a peak height scale up to 8000 RFU.

DNA-based eye colour prediction across Europe with the IrisPlex system. Forensic Science International Genetics 6 (2012) 330-340.

	Brown	Blue	Undefined*	Total
Norway	93	417	37	547
Estonia	90	456	33	579
UK	104	356	38	498
France	392	177	47	616
Italy	321	158	63	542
Spain	396	82	33	511
Greece	463	68	16	547
Total	*1859*	*1714*	*267*	*3840*

* includes perceived green, pupillary rings of alternative colour, other non-brown/blue colours (see Table 1)

Supplementary Table 1: Frequencies of 3 eye colour categories in seven populations from various parts of Europe (EUREYE) as established from eye image analysis.

A new approach for quantifying human pigmentation from digital imagery: improved eye colour phenotyping enhances genetic association and prediction, Draft prepared

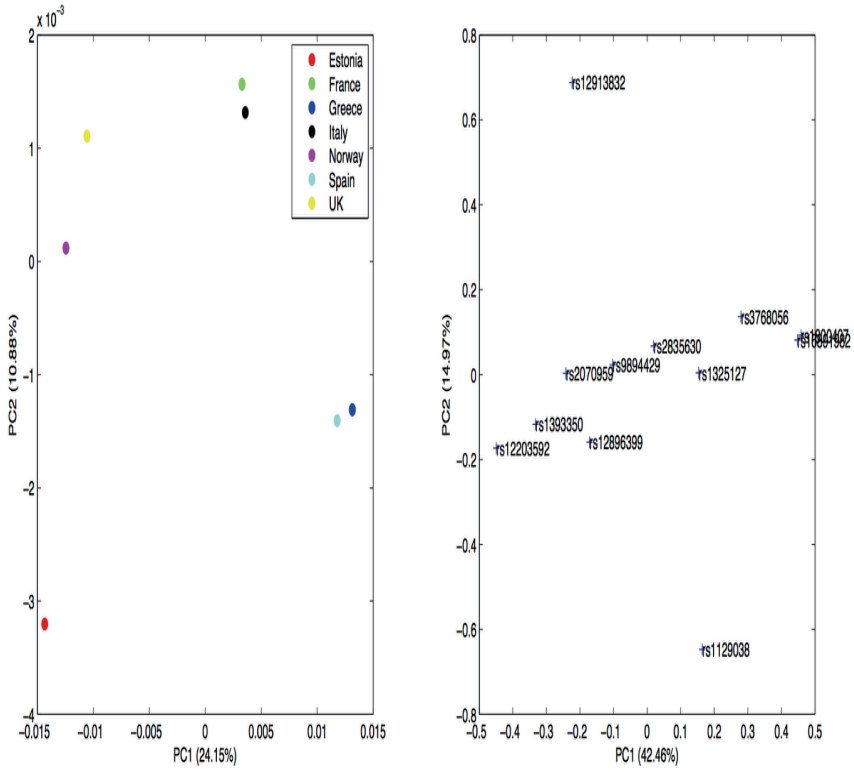


Figure S1: Results from the principal component analysis. Depicted are the population means on the first two principal components applied on individuals as variables.

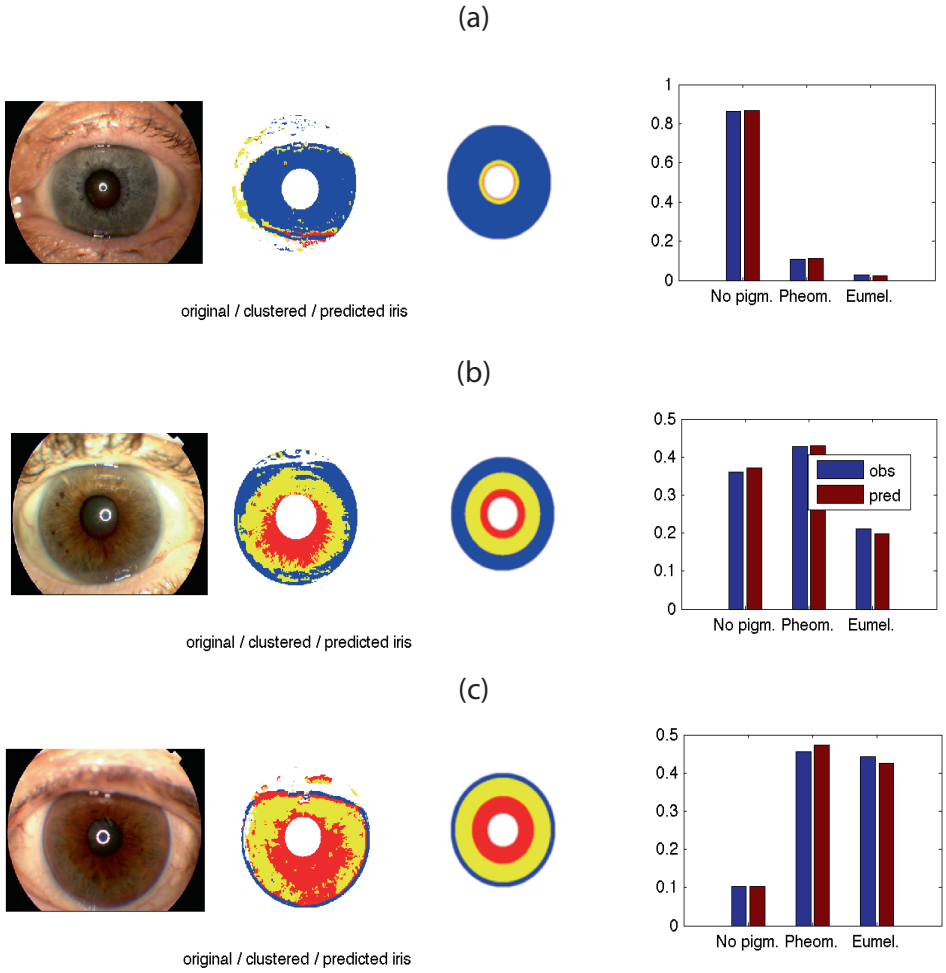


Figure S2: Example of predicted eyes that were manually categorized as blue (panel a), intermediate (panel b) or brown (panel c).

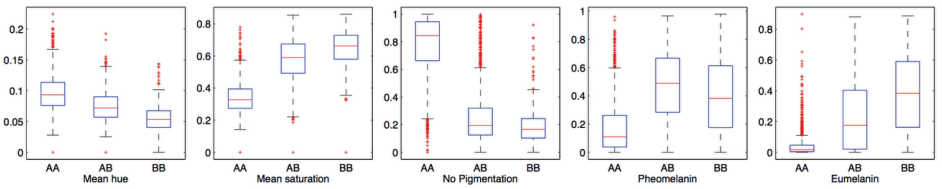
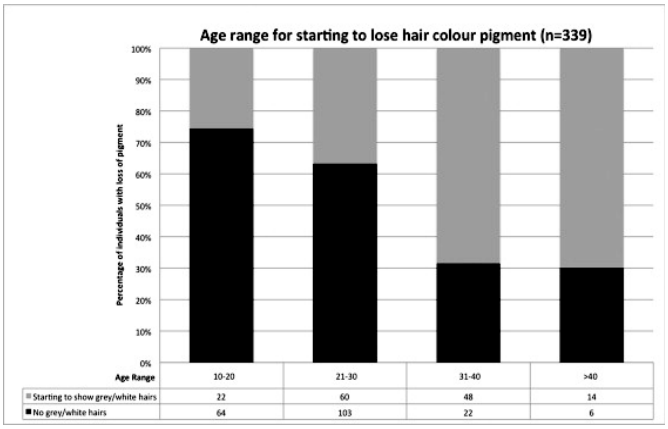
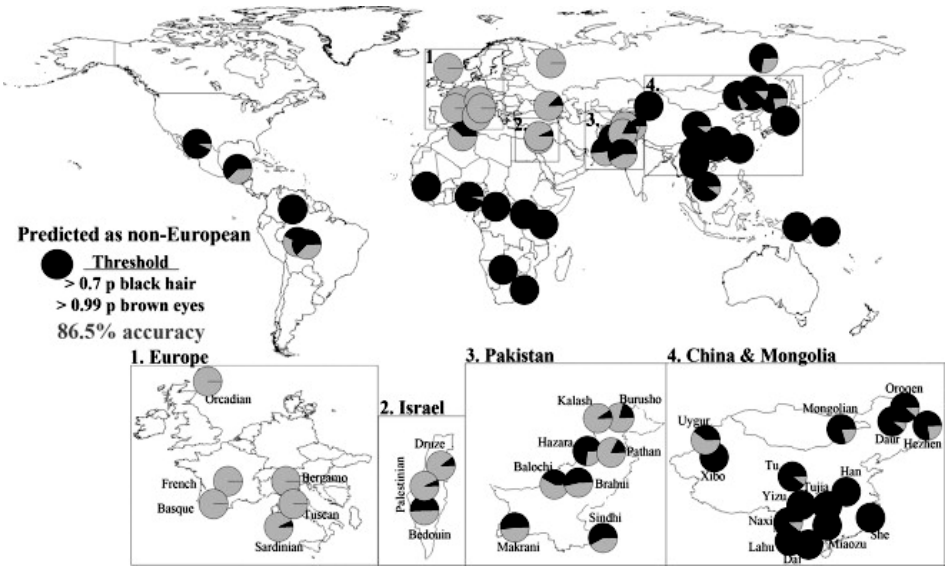


Figure S3: Distribution of quantitative phenotypes grouped by genotype for the most significant SNP rs12913832.

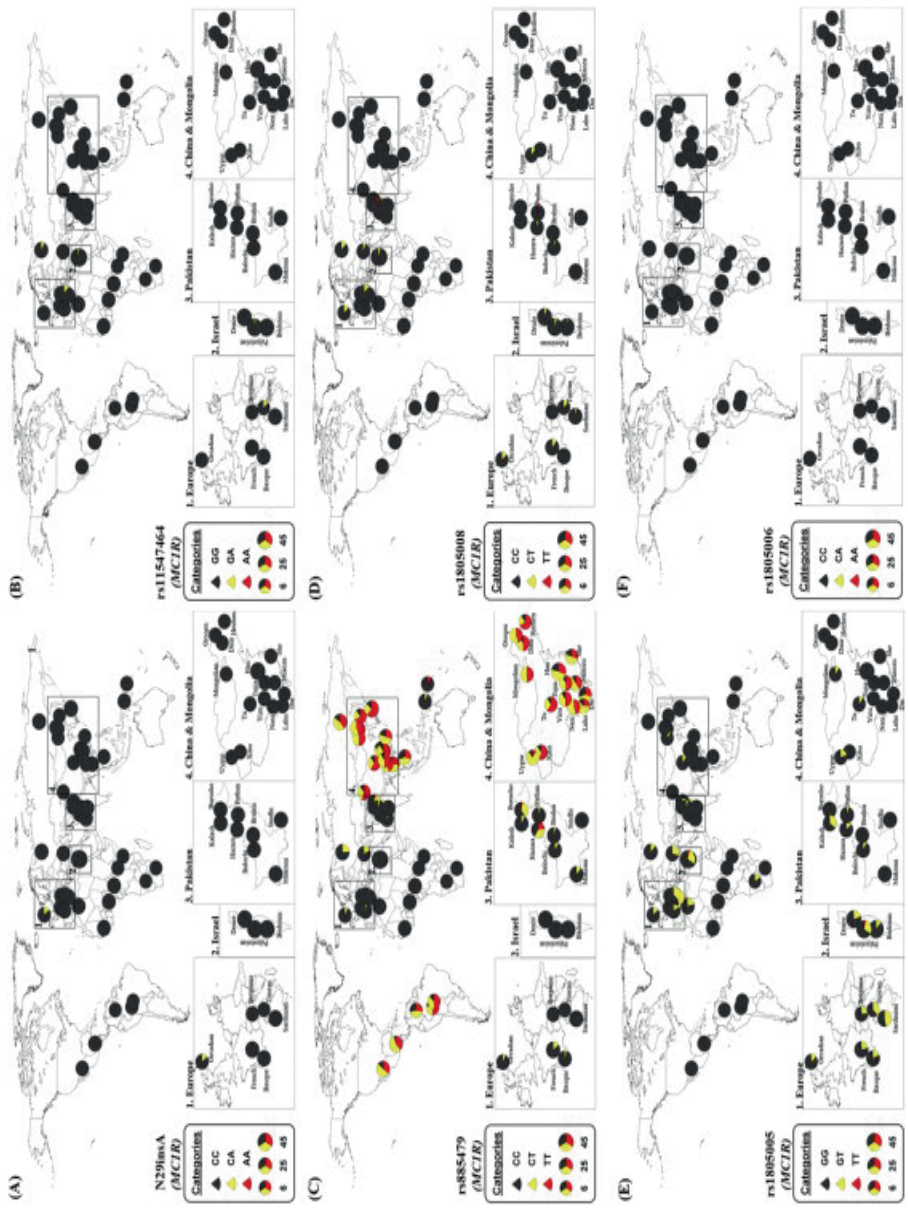
The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. Forensic. Science International: Genetics 7 (2013) 98-115.



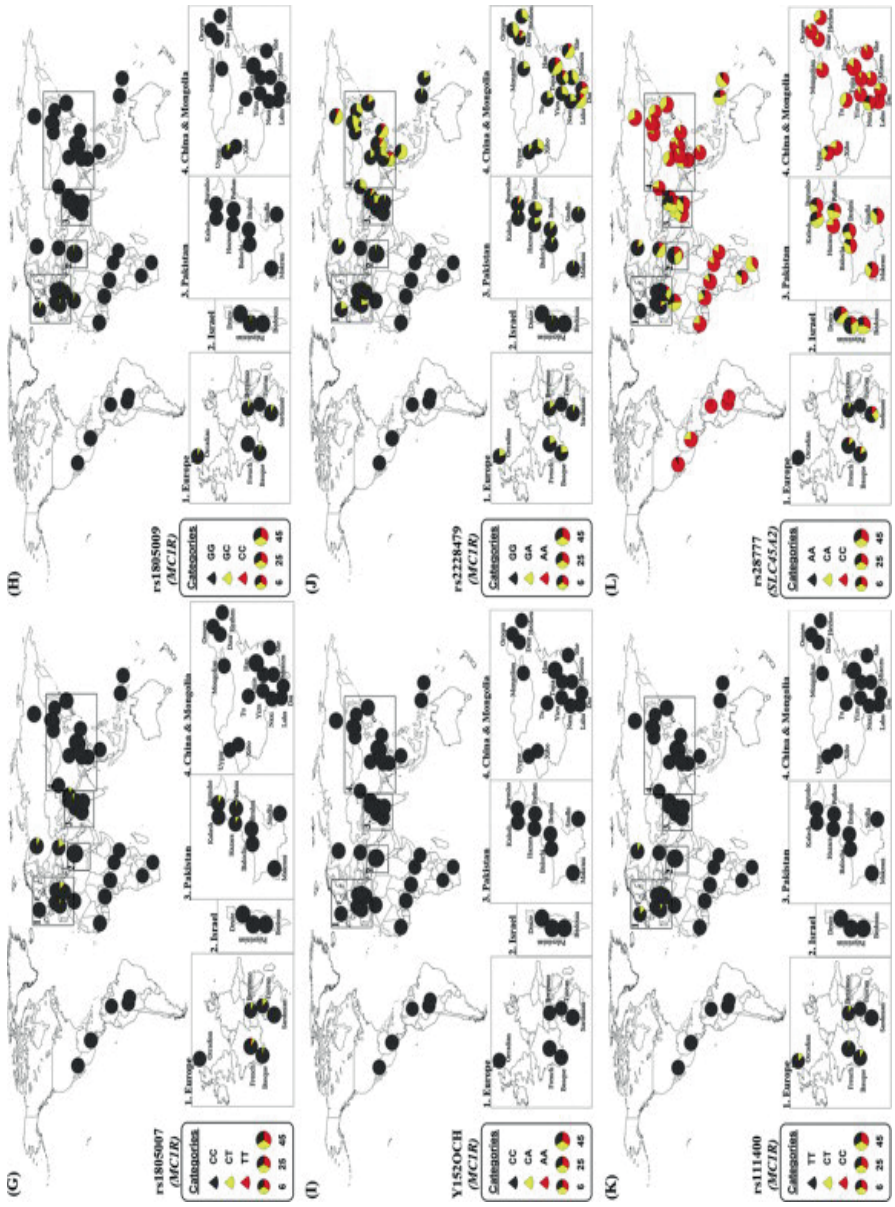
Supplementary Fig. S1. The age range at which individuals first showed signs of grey/white hairs within the Irish population set of $n = 339$. The black colour shows the percentage within the group that did not show signs of hair



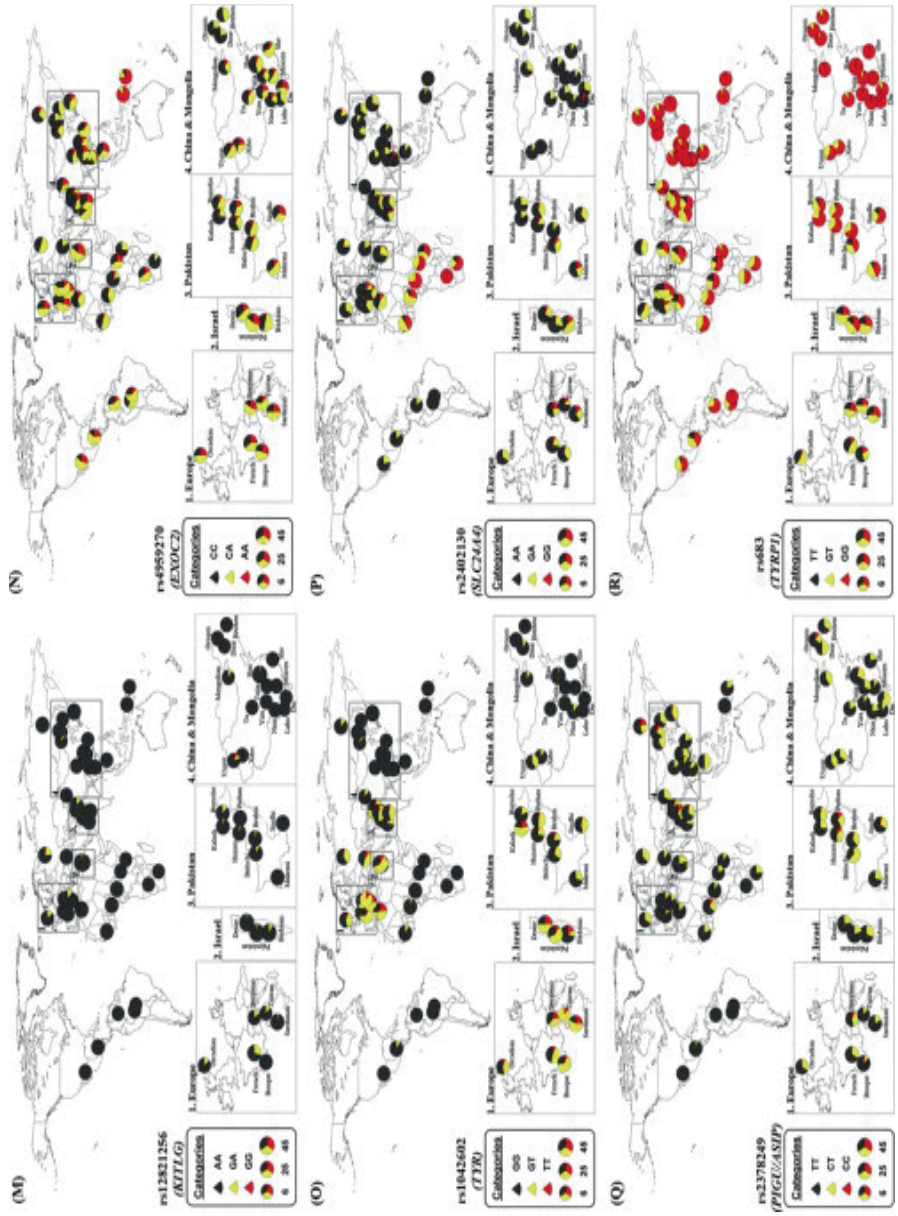
Supplementary Fig. S2. Worldwide survey on how to infer European (including nearby regions) versus non-European (excluding nearby regions) bio-geographic ancestry of a brown eyed, black haired person based on the threshold combination of black hair prediction probability >0.7 and brown eye colour prediction probability >0.99 using the HGDP-CEPH H952 set.



Supplementary Fig. S3. Worldwide distribution of the alleles from the DNA insertion N29insA, and SNPs rs11547464, rs885479, rs1805008, rs1805005, and rs1805006 all from the MC1R gene using the HGDP-CEPH H952 set.



Supplementary Fig. S4. Worldwide distribution of the alleles from the SNPs rs1805007, rs1805009, Y152OCH, rs2228479, rs111400 all from the MC1R gene, and the rs28777 alleles from the *SLC45A2 (MATP)* gene using the HGDP-CEPH H952 set.



Supplementary Fig. S5. Worldwide distribution of the alleles from the SNPs rs12821256 (KITLG), rs4959270 (EXOC2), rs1042602 (TYR), rs2402130 (SLC24A4), rs2378249 (PIGU/ASIP) and rs683 (TYRP1) using the HGDP-CEPH H952 set.

Europe	Total per population	Hair >0.7p black hair	including >.99p brown eye	% Non European calls with threshold
Adygei	17	5	2	12
French	28	3	0	0
French_Basque	23	2	0	0
North_Italian	13	0	0	0
Orcadian	15	0	0	0
Russian	25	0	0	0
Sardinian	28	7	2	7
Tuscan	8	0	0	0
Non-Europe				
Balochi	24	21	10	42
Bantu_N.E.	11	11	11	100
Bantu_S.E.	8	8	8	100
Bedouin	45	44	23	51
Biaka_Pygmies	23	23	23	100
Brahui	24	21	13	54
Burusho	25	13	5	20
Cambodians	10	10	9	90
Colombians	7	7	7	100
Dai	10	10	10	100
Daur	10	10	9	90
Druze	41	17	4	10
Han	43	43	43	100
Hazara	22	21	16	73
Hezhen	9	9	7	78
Japanese	29	29	29	100
Kalash	23	10	2	9
Karitiana	14	13	6	43
Lahu	8	8	8	100
Makrani	25	22	13	52
Mandenka	22	22	22	100
Maya	21	20	13	62
Mbuti_Pygmies	12	12	12	100
Miaozu	10	10	10	100
Mongola	10	10	8	80
Mozabite	29	21	11	38
NAN_Melanesian	11	11	11	100
Naxi	9	9	8	89
Oroqen	9	9	8	89
Palestinian	45	15	3	7
Papuan	17	17	17	100
Pathan	24	16	4	17
Pima	14	14	13	93
San	6	6	6	100
She	10	10	10	100
Sindhi	24	22	14	58
Surui	8	8	5	63
Tu	10	10	9	90
Tujia	10	10	10	100
Uygur	10	7	4	40
Xibo	9	9	9	100
Yakut	25	25	18	72
Yizu	10	10	10	100
Yoruba	22	21	21	95

Supplementary Table S1. Frequency of individuals called non-European in the 51 populations from the HGDP-CEPH H952 set when using black hair colour probabilities >0.7 on its own and in conjunction with brown eye colour probabilities >0.99. Includes the percentage ability to differentiate between a black haired brown-eyed European from a non-European with black hair and brown eyes per population.

Supplementary Table S2. Interactive HIrisPlex prediction tool for hair and eye colour: an easy to use Excel macro to input the minor alleles that are generated from the HIrisPlex genotypes. The output of the tool gives the individual probabilities of the four hair colour categories (Black, brown, red and blond), two hair colour shade categories (light and dark), and three category probabilities for eye colour (blue, intermediate and brown) given its HIrisPlex genotype and based on a prediction model obtained from 1243 Polish, Irish and Greek individuals. For accurate interpretation of hair colour and shade prediction probabilities and to derive the final most likely individual hair colour category see prediction guide in Fig. 4. For accurate interpretation of eye colour prediction probabilities and to derive the final most likely individual eye colour category see recommendations described in Walsh et al. [19].

Supplementary Table S3. Prediction calls of the 308 test set of individuals, includes HIrisPlex probabilities for hair colour categories (including hair shade) and the final prediction call for hair colour (considering colour and shade based on the guide in Fig. 4) as well as eye colour prediction accuracies based on our recommendations described in Walsh et al. [19].

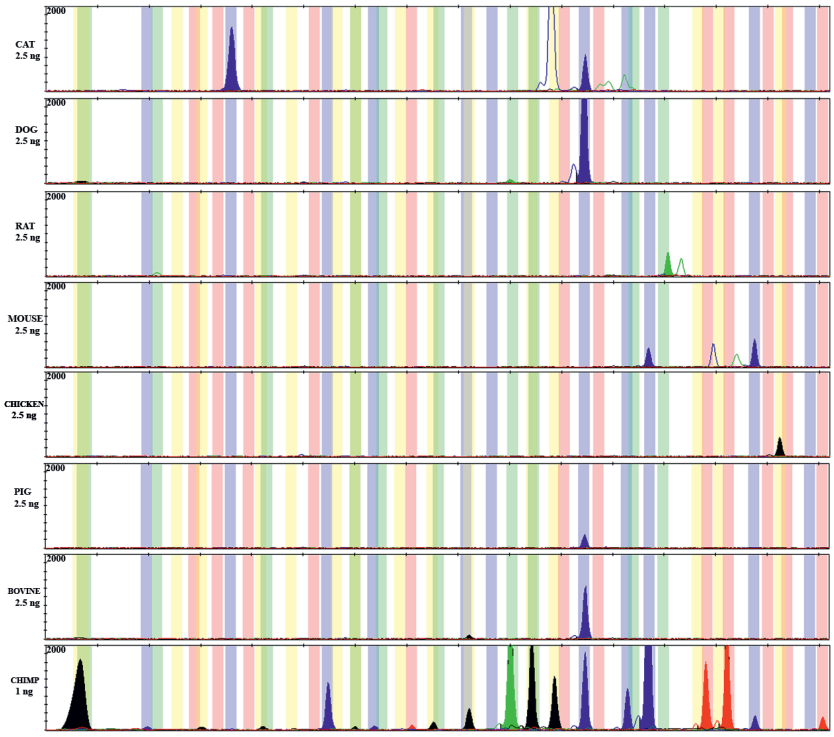
Both found online at http://www.sciencedirect.com/science/article/pii/S1872497312001810

Developmental Validation of the HIrisPlex System: DNA-based eye and hair colour prediction for forensic usage. Draft prepared for submission to Forensic Science International: Genetics.

Supplementary Figure 1: HIrisPlex prediction guide on how to interpret individual hair colour and hair shade probabilities as derived from the HIrisPlex prediction tool taken from our previously published paper [25]. D-brown stands for dark brown and l-brown stands for light brown.

Supplementary Table 3: Interactive macro for HIrisPlex eye and hair colour, including shade prediction probabilities.

Both Found online at
http://www.sciencedirect.com/science/article/pii/S1872497312001810



Supplementary Figure 2: Human specificity testing of the HIrisPlex assay for each SNP on several animal species tested. The presence or absence of a SNP peak is highlighted > 50 RFU. Scale is set to 2000 RFU.

Type	Concentration	Site 1	Site 2 & comments	Site 3 & comments	Site 4 & comments	Site 5 & comments
Saliva	1000 pg	FP	FP	FP	FP	FP
Buccal	800 pg	FP	FP	X Partial profile (20/24) D/O at SNP 1,5,6,7	FP	FP
Saliva	1100 pg	FP	FP	FP	FP	X D/O T allele at SNP 5
Buccal	450 pg	FP	FP	FP	FP	FP
Saliva	1050 pg	FP	FP	FP	FP	FP
Buccal	200 pg	FP	FP	FP	FP	FP
Buccal	50 pg	FP	FP	X Partial profile D/O allele SNP 1	X Partial profile 19/24 D/O allele SNP 1,15,17,22,24	X D/O of C allele
Saliva	137 pg	FP	FP	X Partial profile (6/24) D/O in all but SNP 13,18,20,21,22,23	FP	FP
Buccal	870 pg	FP	FP	FP	FP	FP
Saliva	7000 pg	FP	FP	FP	FP	FP
Buccal	75 pg	FP	FP	FP	FP	FP
Buccal	40 pg	FP	X D/O allele at SNP 22	FP	FP	X D/O of C allele at SNP 8
Buccal	9000 pg	FP	FP	FP	FP	FP
Buccal	16000 pg	FP	FP	FP	FP	FP
Buccal	148 pg	FP	FP	FP	FP	FP
Frozen Blood	500 pg	FP	X Partial profile 23/24 D/O SNP 16	X Drop In of A allele at SNP 1	FP	FP
Buccal	250 pg	FP	FP	FP	FP	FP
Frozen Blood	7000 pg	FP	FP	FP	FP	FP
Saliva	4000 pg	FP	FP	FP	FP	FP
Saliva	350 pg	FP	FP	FP	FP	FP
Saliva	1900 pg	FP	FP	FP	FP	FP
Control DNA	70 pg	FP	FP	FP	FP	FP
Mucus	175 pg	FP	FP	FP	FP	FP
Dried Saliva	80 pg	FP	FP	FP	FP	FP
Trace DNA	79 pg	FP	X Partial Profile, Drop In of T allele at SNP 3 & 4, D/O allele SNP 24	FP	FP	Loss of entire sample due to error
Control DNA	94 pg	FP	FP	FP	FP	FP
Control blank	-	NP	-	-	-	-
Control blank	-	NP	-	-	-	-
Saliva	10000 pg	FP	FP	FP	FP	FP
Cell line	320 pg	FP	FP	FP	FP	FP
				* several samples evaporated due to faulty lids		

Supplementary Table 1: Result of concordance testing from five laboratories including profile interpretation and comments. X indicates an incorrect genotype and drop out (d/o) was called if an allele or full locus was missing from the concordance comparison.

Sample	Type	Simulated casework scenario	DNA amount [pg]	Profile quality	Brown	Red	Black	Blond	Light	Dark	Blue	Int	Brown	Analyst interpretation	Correct/Incorrect on source and prediction?
1	Frozen blood	Single source	4200	good profile	0,131	0,004	0,102	0,763	0,897	0,103	0,870	0,076	0,053	single source, dark blond hair, blue eyed individual	Correct on both
2	Frozen blood	Single source	3000	good profile	0,087	0,000	0,910	0,003	0,006	0,994	0,000	0,006	0,994	single source, light blond hair, blue eyed individual	Correct on both
3	Dried blood	Dried on glass; single source	130	good profile	0,048	0,098	0,009	0,845	0,991	0,009	0,966	0,027	0,007	single source, black hair, brown eyed individual	Correct on both
4	Semen	Single source	500	good profile	0,131	0,004	0,102	0,763	0,897	0,103	0,870	0,076	0,053	single source, dark blond hair, blue eyed individual	Correct on both
5	Saliva	Single source	240	good profile	0,129	0,006	0,029	0,837	0,962	0,038	0,950	0,030	0,020	single source, light to dark blond hair, blue eyed individual	Correct on both
6	Vaginal secretions	Single source	3000	good profile	0,861	0,089	0,000	0,050	1,000	0,000	0,944	0,049	0,007	single source, brown hair, blue eyed individual	Correct on both
7	Saliva	Single source	7600	good profile	0,000	1,000	0,000	0,000	0,913	0,087	0,306	0,142	0,552	single source, red hair, brown to intermediate eyed individual	Correct on both
8	Semen & blood	Mixture: semen & blood	1960	possible mixture										possible mixture present, no brown eye individual in mixture, unsure of hair colour due to mixture	Correct on source
9	Saliva	Single source	11900	good profile	0,223	0,574	0,052	0,151	0,833	0,167	0,505	0,323	0,172	single source,brown red auburn hair, blue to intermediate eyed individual	Correct on source, incorrect on eye colour
10	Mucus	Single source	5390	good profile	0,137	0,004	0,069	0,790	0,921	0,079	0,870	0,076	0,053	single source, dark blond hair, blue eyed individual	Correct on both
11	Saliva	Dried on glass, single source	1520	some low unbalanced peaks in profile possible mixture										unsure of both hair and eye as possible mixture	Incorrect on multiple source
12	Saliva & blood	Mixture: saliva & blood	20000	possible mixture as unbalanced peaks seen										possible mixture present, no brown eye individual in mixture, unsure of hair colour due to mixture	Correct on source
13	Hair	Anagen hair extraction	76	very low sample, quite unbalanced	0,242	0,004	0,068	0,686	0,909	0,091	0,870	0,076	0,053	single source, light brown hair, blue eyed individual	Correct on both
14	Touched item	Trace DNA: single source	50	low profile some unbalanced peaks	0,270	0,000	0,643	0,086	0,085	0,915	0,024	0,083	0,892	single source, dark brown to black hair, brown eyed individual	Correct on both
15	Touched item	Trace DNA: single source	10	hardly no profile, too little DNA											-
16	Mucus & saliva	Mixture: mucus & saliva	18700	possible mixture										unsure of both hair and eye as possible mixture	Correct on source
17	Touched item	Trace DNA: single source	130	good profile	0,261	0,001	0,297	0,441	0,573	0,427	0,306	0,142	0,552	single source, dark brown hair, brown eyed individual	Correct on both
18	Frozen blood	Intentional heme inhibition (2%)	20	good profile	0,131	0,004	0,102	0,763	0,897	0,103	0,870	0,076	0,053	single source, dark blond hair, blue eyed individual	Correct on both
19	Frozen blood	Intentional heme inhibition (10%)	10	very low profile or inhibition											-
20	Touched item	Arm swab: single source	58	low profile	0,098	0,008	0,037	0,858	0,959	0,041	0,950	0,030	0,020	single source, light to dark blond hair, blue eyed individual	Correct on both
21	Saliva	single source	21000	high input good profile	0,076	0,000	0,920	0,003	0,002	0,998	0,000	0,006	0,994	single source, black hair, brown eyed individual	Correct on both
22	Vaginal secretions & semen	Mixture: vaginal swab & semen	31000	possible mixture										unsure of both hair and eye as possible mixture	Correct on source
23	Saliva & semen	Mixture: saliva & semen	1360	possible mixture										unsure of both hair and eye as possible mixture	Correct on source
24	Vaginal swab & blood	Mixture: vaginal swab & blood	14360	good profile	0,053	0,074	0,010	0,863	0,995	0,005	0,962	0,025	0,013	single source, light blond hair, blue eyed individual	Incorrect on single source therefore profile incorrect
25	Touched item	Trace DNA: multiple source	10	unbalanced peaks, possible mixture										unsure of both hair and eye as possible mixture	Correct on source

Supplementary Table 2: Simulated casework samples examined in a blind proficiency testing where only DNA concentrations, without sample type or preparation information, were provided to the analyst, also including comments by analyst and accuracy of HIrisPlex profiles

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a truly wonderful friend, you have always given me the support I needed to get through anything, you are more than my best friend girl, you are my sister.

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LIST OF PUBLICATIONS

2010

F. Liu, A. Wollstein, P.G. Hysi, G.A. Ankra-Badu, T.D. Spector, D. Park, G. Zhu, M. Larsson, D.L. Duffy, G.W. Montgomery, D.A. Mackey, **S. Walsh**, O. Lao, A. Hofman,, F. Rivadeneira, J.R. Vingerling, A.G. Uitterlinden, N.G. Martin, C.J. Hammond, M. Kayser, Digital quantification of human eye color highlights genetic association of three new loci. **PLoS Genet**. 2010 6: e1000934.

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S. Walsh, F. Liu, K. N. Ballantyne, M. van Oven, O. Lao, M. Kayser, IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. **Forensic Science International Genetics** 5 (2011) 170-180.

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2013

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J.Draus-Barini, **S.Walsh**, E.Pośpiech, T.Kupiec1, H.Głąb, W.Branicki & M. Kayser. Bona fide colour: DNA prediction of human eye and hair colour from ancient and contemporary skeletal remains. **Investigative Genetics 2013** 4(3).

PHD-PORTFOLIO

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PhD training

Name	Year	Workload
General research skills		
Safely working in the Laboratory	2012	1 day
Research courses		
Analysis of microarray gene expression data	2009	4 days
Mathworks training on Matlab fundamentals	2009	3 days
SPSS: Statistic course	2009	2 days
Next Generation Sequencing course	2009	3 days
SNP course VI	2009	5 days
Seminars and workshops		
FGCN yearly seminars	2009-2012	4 days
PhD workshop Bruges	2009	5 days
NFI Seminar	2010	1 day
PhD workshop Cologne	2010	5 days
NFI Seminar	2012	1 day
Local Presentations		
Departmental presentations: IrisPlex – eye colour prediction	2009	1 day
Phd Workshop Poster: IrisPlex for eye colour prediction	2010	1 day
FGCN meeting: IrisPlex eye colour prediction across Europe	2011	1 day

NFI Seminar: HIrisPlex: eye and hair colour prediction
2012 1 day

International conferences and presentations

International Society of Forensic Genetics (ISFG), Vienna, Austria

Presentation: The HIrisPlex system: simultaneous prediction of both hair and eye colour from DNA 2011 5 days

International symposium on human identification (ISHI), Washington DC, USA
Workshop presentation: Current FDP Tools: Prediction of Eye and Hair Colour 2011 4 days

Visiting Scientist to the Office of the Chief Medical Examiners Office (OCME), New York, USA
Presentation: HIrisPlex for eye and hair colour prediction
2011 1 day

European Academy of Forensic Sciences (EAFS), Den Haag, NL
Presentation: The HirisPlex System: simultaneous prediction of both hair and eye colour from DNA 2012 5 days

International symposium on human identification (ISHI), Nashville TN, USA
Presentation: HIrisPlex: DNA Test System for Eye & Hair Colour prediction
2012 4 days

ABOUT THE AUTHOR

Susan Walsh was born on July 8th, 1982 in Cork, Ireland. She graduated from secondary school at Loreto Secondary School Youghal in 2001. In the same year she began her studies at University College Cork, Ireland where she obtained a BSc. in Biochemistry for which she received an honours degree in 2005. Following her BSc. she immediately went on to pursue a MSc. in DNA profiling from the University of Central Lancashire in the UK. After obtaining her MSc. in 2006, she decided to do some travelling and ended up in Sydney, Australia where she worked at the University of Sydney in a DNA sequencing facility until 2009. In January 2009 she moved back to Europe to begin her PhD studies at the Department of Forensic Molecular Biology, Rotterdam, the Netherlands. Under the guidance of her supervisor, Professor Manfred Kayser, she has successfully published in several peer-reviewed journals and has presentend her work at several interational conferences worldwide. From 2009 – 2013 she has fully established her name within the forensic arena and is known as a specialist in the field of forensic DNA phenotyping. Susan is married to Michael J. Harman and currently resides in Connecticut, USA.