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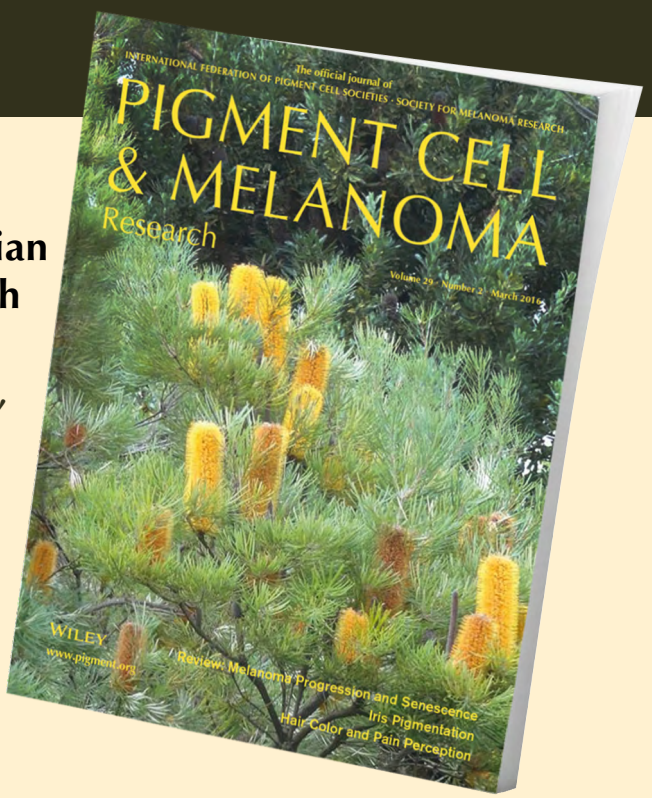

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Summary

In this study, we present a new quantitative method to measure iris colour based on high-resolution photographs. We applied this method to analyse iris colour variation in a sample of individuals of East Asian, European and South Asian ancestry. We show that measuring iris colour using the coordinates of the CIELAB colour space uncovers a significant amount of variation that is not captured using conventional categorical classifications, such as 'brown', 'blue' or 'green'. We tested the association of a selected panel of polymorphisms with iris colour in each population group. Six markers showed significant associations with iris colour in the European sample, three in the South Asian sample and two in the East Asian sample. We also observed that the marker *HERC2* rs12913832, which is the main determinant of 'blue' versus 'brown' iris colour in European populations, is also significantly associated with central heterochromia in the European sample.

Introduction

Iris colour in humans is a complex trait that is primarily the product of differences in the structure and organization of the eye. The typical iris consists of five layers: the iris pigment epithelium (IPE), the sphincter and dilator muscles, the stromal layer (SL) and the anterior border layer (ABL) (Eagle, 1988; Oyster, 1999; Sturm and Larsson, 2009). The IPE is derived from the neuroectoderm and contains many large, densely packed spherical melanosomes (Eagle, 1988; Prota et al., 1998; Wilkerson et al., 1996). The amount and composition of melanin in this layer is similar in all healthy humans as the primary purpose of the IPE is to protect the retina and absorb excess light (Wilkerson et al., 1996). As a result, variation in this layer does not contribute significantly to normal iris pigmentation variation. In contrast, the melanosomes in the SL and ABL, which originate from the neural crest, are smaller, ovoid-shaped and differ widely between individuals (Imesch et al., 1996; Prota et al., 1998; Sturm and Larsson, 2009). It is presently believed that the majority of iris pigmentation variation can be directly linked to the amount and type of melanin found in these two layers, with darker eyes having more melanin and a higher eumelanin/pheomelanin ratio than lighter eyes (Peles et al., 2009; Wakamatsu et al., 2008; Wielgus and Sarna,

Significance

Using quantitative measures of iris colour uncovers a significant amount of variation that is not captured by the traditional categorical classifications and is an ideal tool to explore the genetic architecture of iris colour in human populations.

2005). However, there are a number of other factors that can also influence iris colour, including the depth of the SL, the organization of the extracellular components in the SL and ABL, the thickness and curvature of the cornea, the presence of structures (e.g. pigment spots, Wolfflin nodules) in the iris and the amount of melanin found in the SL relative to the ABL (Baranoski and Lam, 2007; MacKey et al., 2011; Prota et al., 1998; Wielgus and Sarna, 2005).

Iris colour shows a very distinct global distribution. Across Europe, and to a lesser degree North Africa, the Middle East and Central Asia, irises show extensive pigmentation variation and range from light blues to greens to browns (Donnelly et al., 2012; Eiberg et al., 2008). Many irises in these regions also show central heterochromia, where there is a band of colour around the pupil that differs from the rest of the eye. Throughout the rest of the world, however, iris colour appears to be much more homogenous and is primarily limited to varying shades of brown. At present, the genetic basis of iris colour has been extensively studied in populations of European ancestry. The majority of variation between blue and brown eye colour has been attributed to the marker *HERC2* rs12913832, which is located in a highly conserved region of the genome that is believed to regulate the transcription of the nearby pigmentation gene, *OCA2* (Eiberg et al., 2008; Kayser et al., 2008; Sturm et al., 2008). It has been suggested that this polymorphism is located in the middle of a helicase-like transcription factor (HLTF) binding site (Sturm et al., 2008; Visser et al., 2012). When the ancestral A allele is present, HLTF (a chromatin remodelling protein) is able to recognize the sequence and begin unwinding the chromatin. This exposes sequences for other regulatory proteins and promotes the synthesis of *OCA2*. When the derived G allele is present, however, there is reduced recruitment of HLTF and *OCA2* transcription is limited. Although *HERC2* rs12913832 may be the primary determinant of global iris colour variation, a number of other markers have also been identified that have a more subtle effect on iris pigmentation. These include polymorphisms in *OCA2*, *SLC45A2*, *SLC24A4*, *TYRP1* and *IRF4* (Graf et al., 2005; Rebbeck et al., 2002; Sturm et al., 2008; Sulem et al., 2007; Walsh et al., 2011). The identification of markers associated with iris colour in European populations has allowed several forensic science groups to develop algorithms that are capable of predicting iris colour from DNA samples obtained at crime scenes and from other unidentified persons (Ruiz et al., 2014; Spichenok et al., 2011; Walsh et al., 2011).

Despite our growing understanding of the genetic basis of iris pigmentation in European populations, very few groups have attempted to look at eye colour in populations of non-European ancestry. Although brown eye colour does dominate in regions outside of Europe, there is much variation within these browns and they can range from light reddish-yellows to dark brownish-blacks. In recent years, a number of methods have been developed that allow researchers to characterize iris colour quantitatively. Liu et al. (2010) obtained hue and saturation values from iris photographs, with hue being used to represent the type of melanin and saturation being used to represent the amount of melanin in the iris. Andersen et al. (2013) developed a PIE (Pixel Index of the Eye) score that was computed using the number of pixels labelled brown and the number of pixels labelled blue in photographs of the iris. Recently, we extracted a colour measurement in CIE 1976 L*a*b* (CIELAB) colour space from a 256 by 256 pixel square isolated using high-resolution photographs of the iris (Edwards et al., 2012). These methods go beyond the traditional categorical classification systems that have been used in the past (i.e. 'green' 'blue' 'brown') and provide a means of studying iris colour variation in populations with more homogenously coloured irises. However, thus far, they have not yet been broadly applied to global populations.

Using quantitative methods to study the genetic basis of iris colour in populations of non-European ancestry would greatly contribute to our overall understanding of global iris colour variation. At present, we have a limited understanding of the markers responsible for intermediate iris colours. This is reflected in the fact that most eye colour prediction algorithms perform poorly and inconsistently when looking at intermediately coloured eyes (Dembinski and Picard, 2014; Pneuman et al., 2012; Yun et al., 2014). In addition, identifying novel variants associated with iris colour variation in populations of non-European ancestry would provide valuable information about the genetic basis of skin and hair colour in these regions, given the known pleiotropic effects of some genetic markers on pigmentary traits. Studying iris colour may also be of biomedical interest. Eye colour has been found to be associated with a number of ocular disorders (Mitchell et al., 1998; Wakamatsu et al., 2008). The most well known of these is perhaps age-related macular degeneration (AMD). This disease, which primarily affects older adults, results from damage to the retina and can lead to vision loss and blindness. Age-related macular degeneration has been strongly linked to population-specific genetic effects (Frank et al., 2000). However, within populations, eye colour may be one of the determinants of AMD. In European samples, it has been found that individuals with blue eyes have a higher risk of developing AMD (Frank et al., 2000; Mitchell et al., 1998). Similarly, the incidence of primary angle-closure glaucoma in East Asian populations may be associated with variation in brown iris colour. A recent study found that individuals with dark brown eyes in a Malaysian population living in Singapore had a narrower angle closure than individuals with light brown eyes (Sidhartha et al., 2014). Thus, it is not only necessary to investigate the genes responsible for the difference between blue, brown and intermediate phenotypes in European popula-

tions, but also to look at the genes that modulate brown iris pigmentation in other regions as well.

In this study, we have three primary goals: (i) to improve our quantitative method of measuring iris colour to better capture iris colour variation in populations of diverse ancestry, (ii) to look at the association between putative pigmentation markers and iris colour in a sample of European, East Asian and South Asian ancestry and (iii) to look at the association between pigmentation markers and central heterochromia within the European and South Asian samples.

Results

In total 474 East Asians, 624 Europeans and 367 South Asians were included in the study. Six East Asians, four Europeans and four South Asians were unable to remove their contact lenses for the photograph and were excluded from the analysis. Two participants of South Asian descent were removed from the study because they had an obstructed iris wedge. No iris obstruction could be observed in any of the other participants. A final participant of South Asian descent was excluded because the participant reported having a form albinism that affected ocular pigmentation.

Inter- and intrarater reliability measurements were excellent for all four colour space measurements (Table 1). Substantial dispersion across CIE 1976 L*a*b* (CIELAB) colour space could be seen in all three populations (Figure 1 and Figure S1). Blue eyes tend to show high L* values and negative a* and b* values, while brown eyes have low L* values and high a* and b* values. Due to the arc shape in which irises are distributed across CIELAB colour space, dark brown eyes have a lower L*, a* and b* values than lighter brown eyes (Figure 1 and Figure S1). Green eyes tend to have a* values that are intermediate between blue and brown irises. The greatest amount of iris colour variation was found in the European sample. However, there was also extensive variation in the South and East Asian groups. The average, minimum and maximum L*, a*, b* and ΔE measurements for all 1448 participants is presented in Table 2. There is a noticeable difference between the two camera bodies in the L* dimension, confirming that the bodies have some variation in brightness. As a* and b* are colour dimensions that were designed to be independent from brightness, it is not surprising that there is less variation in these coordinates (McLaren, 1976). However, all three dimensions appear to show somewhat higher values in photographs taken with the first camera compared to those taken with the second camera.

Table 1. Results of the inter- and intra-rater reliability analysis

	Intra-rater intracorrelation coefficient	Inter-rater intracorrelation coefficient
L*	0.999	0.999
a*	0.999	0.997
b*	0.997	0.992
ΔE	0.985	0.985

We report the intracorrelation coefficient for a two-way mixed model for both the inter-rater and intrarater measurements.

In each population, we looked at the association between markers purported to play a role in pigmentation variation and the L*, a* and b* coordinates. In the European and South Asian populations, the association between each of these markers and ΔE was also examined. With the exception of *OCA2* rs74653330 in the East Asian sample, we only looked at polymorphisms that had a minor allele frequency of at least 0.05. For the European population, this included *HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *TYR* rs1393350, *IRF4* rs12203592, *DSCR9* rs7277820, *TYRP1* rs1408799, *NPLOC4* rs9894429, *LYST* rs3768056 and *ASIP* rs6058017. In the East Asian population, this included markers *SLC24A4* rs12896399, *DSCR9* rs7277820, *NPLOC4* rs9894429, *LYST* rs3768056, *ASIP* rs6058017, *OCA2* rs1800414 and *OCA2* rs74653330. Lastly, in the South Asian population this included markers *HERC2* rs12913832, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *SLC24A5* rs1426654, *TYR* rs1393350, *IRF4* rs1126809, *DSCR9* rs7277820, *TYRP1* rs1408799, *NPLOC4* rs9894429, *LYST* rs3768056 and *ASIP* rs6058017. Linkage disequilibrium was low ($r^2 < 0.1$) between all pairs of markers located in the *OCA2/HERC2* region. Most markers showed no deviations from Hardy–Weinberg equilibrium. However, *DSCR9* rs7277820 showed minor deviations in the European ($P = 0.0169$) and South Asian ($P = 0.0473$) samples and *SLC24A5* rs1426654 showed major deviations ($P = 1.199 \times 10^{-5}$) in the South Asian sample. Twenty-five individuals (five East Asians, 10 Europeans and 10 South Asians) were missing genotypes for more than 20% of the polymorphisms and were excluded from the genetic analysis.

As the two camera bodies appeared to show some variation in the L*, a* and b* dimensions, we chose to carry out a meta-analysis on the two data sets. A linear association analysis was first performed in each population to look at the association between each SNP and the L*, a* and b* values for each camera body. In the European and South Asian populations, an additional analysis was carried out for the ΔE measurement. The meta-analysis function in PLINK, using a fixed effects model, was then used to determine the combined significance and effect size in each population.

The results of the meta-analysis in the European population can be found in Table 3a. *HERC2* rs12913832 was strongly associated with the L*, a* and b* dimensions of colour space, with the ancestral A allele decreasing the L* and increasing the a* and b* coordinates. For this marker, the a* and b* dimensions showed significant deviations from additivity, indicating that these

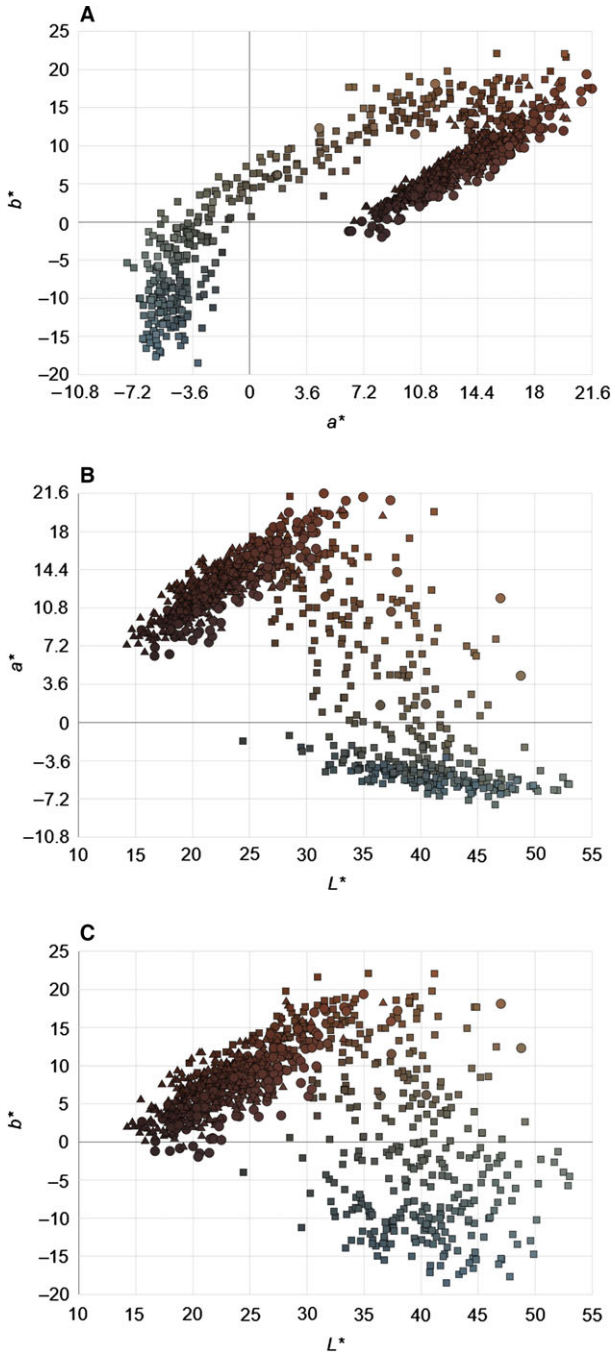

Figure 1. The distribution of irises across the b* and a* (A), a* and L* (B), and b* and L* (C), coordinates of CIE 1976 L*a*b* (CIELAB) colour space for the second camera body. East Asian participants are represented by triangles, European participants are represented by squares, and South Asian participants are represented by circles. The colour inside of each shape is equivalent to the average iris colour of the participant's wedge in CIELAB colour space. Ample dispersion across colour space can be observed in all three populations. Both cameras show very similar distributions across colour space. However, the average L*, a* and b* values from the first camera are higher than those from the second camera. The distribution of irises across colour space for the first camera can be found in Figure S1.

two colour dimensions are best modelled using a dominant/recessive mode of inheritance (e.g. the ancestral A allele is dominant over the derived G allele). In contrast, the L* coordinate showed no significant deviations from additivity. *SLC24A4* rs12896399, *SLC45A2* rs16891982 and *IRF4* rs12203592 were significantly associated with the a* and b* dimensions. For all three markers, the derived alleles decrease both coordinates. Although we had no individuals who were homozygous for the derived genotype in our sample, *OCA2* rs1800407 was significantly associated with an increase in the b* dimensions of colour space. Apart from *HERC2* rs12913832, none of the markers showed any significant deviations from an additive mode of inheritance.

As *HERC2* rs12913832 is known to be the primary variant responsible for controlling the difference between blue and brown eye colour, we ran the analysis again after conditioning for this marker (Table 3b). Most polymorphisms showed similar effects after conditioning. Interestingly, although *OCA2* rs1800407 was no longer associated with the b* dimension of colour space, it was now associated with the L* and a* dimensions, with the derived allele increasing the L* coordinate and decreasing the a* coordinate. Lastly *TYR* rs1393350 was now associated with the L* dimension of colour space, with the derived A allele decreasing this coordinate.

In the South Asian sample, *HERC2* rs12913832 was strongly associated with the L*, a* and b* dimensions of colour space (Table 4a). The derived G allele was associated with an increase in the L* and b* dimensions, and a decrease in the a* dimension. *HERC2* rs12913832 showed significant deviations from additivity for all three dimensions of colour space indicating that it is best modelled using a dominant/recessive mode of inheritance. *SLC45A2* rs1426654 was strongly associated with the L*, a* and b* dimensions of colour space, and each copy of the ancestral allele was responsible for a decrease in all three coordinates. The only other marker associated with iris colour in the South Asian sample was *LYST* rs3768056, with the derived G allele increasing both the a* and b* coordinates. Conditioning on *HERC2* rs12913832 in this population had very little effect on both significance levels and effect size (Table 4b).

In the East Asian population, only *OCA2* rs1800414 and *OCA2* rs74653330 were associated with iris colour variation (Table 5a). *OCA2* rs1800414 had a significant effect on the L*, a* and b* dimensions of colour space, with each copy of the ancestral allele decreasing all three coordinates. Although no participants were homozygous for the derived allele at *OCA2* rs74653330, having one copy significantly increased both the a* and b* coordinates. After conditioning for *OCA2* rs1800414, the effect of *OCA2* rs74653330 became stronger, and this marker was now associated with all three dimensions of colour space (Table 5b). For this marker, the derived A allele

Table 2. Summary statistics for all 1448 participants included in the study

	Regional ancestry	Camera body	Total number	Average age (min, max)	Males (females)	Average L* (min, max)	Average a* (min, max)	Average b* (min, max)	Average ΔE (min, max)
East Asian	China	1	87	21 (18,34)	26 (61)	26.914 (19.525,36.683)	13.674 (8.832,20.391)	8.158 (0.982,17.891)	2.476 (0.638,4.748)
		2	274	22 (18,35)	78 (196)	22.182 (14.239,36.669)	12.9 (7.299,20.010)	7.289 (0.3,18.203)	2.109 (0.063,5.705)
	Japan	1	2	21 (19,23)	1 (1)	27.372 (24.384,30.36)	14.311 (13.202,15.419)	7.329 (6.544,8.115)	1.762 (1.605,1.918)
		2	6	24 (18,29)	1 (5)	21.846 (15.43,26.468)	13.563 (9.845,15.087)	8.714 (5.543,11.411)	2.006 (0.657,2.788)
	Korea	1	21	24 (18,29)	10 (11)	27.055 (22.65,38.532)	13.732 (8.399,17.410)	8.728 (0.932,16.177)	2.251 (0.399,6.519)
		2	52	22 (18,31)	24 (28)	21.034 (15.814,28.17)	12.56 (6.594,18.147)	7.136 (0.098,18.372)	2.203 (0.523,5.199)
	Taiwan	1	2	21 (20,22)	1 (1)	27.08 (23.85,30.31)	12.524 (10.539,14.509)	6.531 (3.354,9.708)	2.607 (2.046,3.168)
		2	7	20 (19,23)	2 (5)	19.798 (16.502,23.522)	10.961 (7.709,13.119)	4.635 (1.36,6.767)	1.881 (1.329,2.868)
	Other	1	4	21 (19,25)	0 (4)	27.43 (24.298,30.477)	13.437 (10.063,16.724)	7.911 (3.51,12.166)	2.992 (2.311,3.861)
		2	13	21 (18,26)	5 (8)	20.44 (14.685,26.547)	11.949 (7.921,16.724)	6 (−0.664,11.902)	1.999 (0.748,3.203)
European	Eastern Europe	1	53	21 (18,35)	24 (29)	45.002 (30.834,62.23)	1.055 (−8.444,18.733)	2.252 (−15.797,20.44)	9.277 (1.114,16.684)
		2	67	23 (18,35)	25 (42)	38.229 (18.899,53.004)	1.663 (−7.734,19.962)	1.795 (−17.65,21.611)	7.671 (0.683,16.153)
	Northern Europe	1	33	23 (19,35)	11 (22)	45.279 (30.857,57.16)	0.43 (−8.225,16.027)	1.828 (−18.61,21.473)	9.129 (3.508,17.378)
		2	74	24 (18,35)	33 (41)	37.97 (25.269,49.584)	−1.5 (−6.961,17.543)	−3.406 (−15.832,22.095)	6.832 (1.702,14.721)
	Southern Europe	1	47	21 (18,27)	16 (31)	38.821 (26.084,55.444)	10.894 (−6.601,22.009)	12.512 (−14.18,22.418)	6.451 (0.796,14.674)
		2	52	22 (18,35)	22 (30)	33.813 (21.294,52.467)	8.942 (−6.713,21.318)	10.045 (−12.828,19.776)	6.4 (0.657,15.402)
	Western Europe	1	3	20 (18,23)	0 (3)	47.903 (41.213,52.606)	−3.663 (−6.977, −1.149)	2.576 (−1.76,4.923)	12.418 (10.731,13.312)
		2	5	24 (19,32)	2 (3)	35.911 (28.341,43.317)	4.705 (−5.882,13.278)	5.376 (−17.356,13.636)	6.456 (1.515,10.712)
	Other	1	99	22 (18,31)	36 (63)	44.622 (27.588,61.609)	1.611 (−8.035,21.032)	1.911 (−18.957,24.087)	8.447 (1.343,19.264)
		2	187	23 (18,35)	76 (110)	38.191 (19.674,52.872)	0.436 (−7.315,19.87)	0.328 (−18.475,22.067)	7.239 (0.509,16.921)
South Asian	Bangladesh	1	5	24 (18,33)	1 (4)	27.792 (22.245,30.599)	12.977 (7.632,15.868)	6.099 (0.481,9.061)	3.257 (2.339,4.588)
		2	12	21 (18,32)	5 (7)	25.107 (19.351,31.789)	13.419 (11.086,16.25)	8.679 (3.468,17.146)	2.663 (0.416,8.207)
	India	1	93	21 (18,32)	32 (61)	29.56 (19.918,50.209)	13.81 (−0.912,22.671)	8.564 (−1.732,20.147)	2.807 (0.908,10.391)
		2	88	21 (18,35)	38 (50)	25.105 (15.533,37.93)	14.16 (6.26,21.256)	8.134 (−1.427,19.367)	2.117 (0.156,7.782)
	Pakistan	1	48	20 (18,24)	15 (33)	30.401 (21.266,50.995)	14.665 (0.429,25.925)	9.595 (−1.2,26.796)	3.204 (0.451,13.486)
		2	36	20 (18,31)	9 (27)	27.53 (18.311,48.769)	14.028 (1.643,19.802)	9.148 (−1.955,18.111)	2.99 (0.561,11.616)
	Sri Lanka	1	23	20 (18,21)	5 (18)	27.698 (18.31,42.544)	12.049 (7.101,18.796)	6.001 (−0.198,15.982)	2.343 (0.247,7.118)
		2	26	20 (18,26)	5 (21)	23.467 (16.704,40.457)	11.5 (1.746,21.599)	4.772 (−1.493,17.479)	2.317 (0.926,9.633)
	Other	1	19	20 (18,23)	6 (13)	32.627 (25.853,47.392)	13.747 (−2.94,18.521)	9.735 (2.136,21.973)	3.437 (0.378,10.773)
		2	10	20 (18,27)	2 (8)	24.451 (18.644,30.629)	14.169 (10.034,19.247)	8.212 (3.967,16.983)	2.577 (1.003,5.219)

We show the sex, average, minimum and maximum age, L*, a*, b* and ΔE values of all 1448 participants included in the analysis (excluded individuals have been omitted from this table) for each of the two camera bodies. Participants are divided both by broad ancestry (East Asian, European and South Asian) and regional ancestry. European participants are divided into regions using the United Nations geoscheme for Europe (http://unstats.un.org/unsd/methods/m49/m49regin.htm). East Asian and South Asian participants are divided by country of origin.

Table 3. Results of the meta-analysis in the European sample (a) before and (b) after conditioning for the effects of *HERC2* rs12913832

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
(a)							
HERC2	rs12913832	GG	L*	GA	***3.907 × 10^{-54}***	***−7.143***	***0.041***
				AA	***1.023 × 10^{-56}***	***−12.586***	***0.023***
			a*	GA	***4.641 × 10^{-216}***	***12.971***	***0.031***
				AA	***5.203 × 10^{-137}***	***17.985***	***0.039***
			b*	GA	***1.872 × 10^{-223}***	***18.343***	***0.071***
				AA	***1.863 × 10^{-96}***	***20.659***	***0.271***
			ΔE	GA	***1.098 × 10^{-05}***	***−1.236***	***0.443***
				AA	***3.667 × 10^{-15}***	***−3.846***	***0.088***
OCA2	rs1800407	GG*	L*	GA	0.796	0.221	0.252
				AA	–	–	–
			a*	GA	0.150	1.542	0.748
				AA	–	–	–
			b*	GA	***0.003***	***4.297***	***0.887***
				AA	–	–	–
			ΔE	GA	0.852	−0.080	0.431
				AA	–	–	–
SLC24A4	rs12896399	GG*	L*	GT	0.066	1.121	0.556
				TT	0.325	0.789	0.648
			a*	GT	***0.004***	***−2.202***	***0.759***
				TT	0.080	−1.759	0.596
			b*	GT	***7.191 × 10^{-04}***	***−3.446***	***0.470***
				TT	***1.758 × 10^{-04}***	***−5.018***	***0.707***
			ΔE	GT	0.949	−0.019	0.718
				TT	0.071	−0.725	0.060
SLC45A2	rs16891982	GG	L*	GC	0.059	−1.610	0.085
				CC	0.230	−3.818	0.026
			a*	GC	***9.655 × 10^{-07}***	***5.139***	***0.170***
				CC	0.050	7.714	0.047
			b*	GC	***2.069 × 10^{-06}***	***6.740***	***0.666***
				CC	0.244	6.100	0.116
			ΔE	GC	0.150	−0.621	0.659
				CC	0.209	−2.039	0.422
TYR	rs1393350	GG*	L*	GA	0.543	−0.349	0.022
				AA	0.029	2.604	0.077
			a*	GA	0.443	−0.556	0.356
				AA	0.261	−1.694	0.725
			b*	GA	0.077	−1.721	0.308
				AA	0.261	−2.258	0.883
			ΔE	GA	0.896	−0.038	0.247
				AA	0.019	1.404	0.026
IRF4	rs12203592	CC*	L*	CT	0.206	0.802	0.791
				TT	0.033	3.381	0.921
			a*	CT	0.008	−2.077	0.473
				TT	**0.003**	**−5.917**	**0.464**
			b*	CT	0.013	−2.624	0.167
				TT	***1.072 × 10^{-04}***	***−10.058***	***0.523***
			ΔE	CT	0.041	0.652	0.967
				TT	0.704	−0.305	0.634
TYRP1	rs1408799	CC	L*	CT	0.790	−0.156	0.238
				TT	0.041	−1.857	0.409
			a*	CT	0.245	0.858	0.425
				TT	0.007	3.091	0.890
			b*	CT	0.248	1.151	0.739
				TT	0.083	2.669	0.447
			ΔE	CT	0.154	−0.420	0.381
				TT	0.096	−0.763	0.134
NPLOC4	rs9894429	CC*	L*	CT	0.109	−1.035	0.305
				TT	0.686	−0.321	0.359

Table 3. (continued)

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
			a*	CT	0.890	0.112	0.623
				TT	0.117	−1.564	0.518
			b*	CT	0.845	−0.213	0.252
				TT	0.040	−2.755	0.549
			ΔE	CT	0.489	0.225	0.245
				TT	0.167	0.554	0.530
LYST	rs3768056	AA*	L*	AG	0.151	−0.842	0.762
				GG	0.438	1.010	0.015
			a*	AG	0.097	1.226	0.354
				GG	0.814	0.387	0.142
			b*	AG	0.056	1.890	0.594
				GG	0.904	−0.267	0.095
			ΔE	AG	0.165	−0.408	0.462
				GG	0.875	−0.103	0.006
ASIP	rs6058017	AA	L*	AG	0.185	−0.906	0.296
				GG	0.670	−0.951	0.391
			a*	AG	0.047	1.695	0.448
				GG	0.393	2.396	0.480
			b*	AG	0.012	2.887	0.536
				GG	0.309	3.789	0.248
			ΔE	AG	0.391	−0.294	0.023
				GG	0.065	2.079	0.551
(b)							
OCA2	rs1800407	GG*	L*	GA	***1.767 × 10^{-07}***	***3.552***	***0.498***
				AA	–	–	–
			a*	GA	***2.203 × 10^{-09}***	***−3.835***	***0.872***
				AA	–	–	–
			b*	GA	0.013	−2.389	0.624
				AA	–	–	–
			ΔE	GA	0.082	0.736	0.772
				AA	–	–	–
SLC24A4	rs12896399	GG*	L*	GT	0.111	0.909	0.200
				TT	0.457	0.555	0.520
			a*	GT	0.007	−1.902	0.311
				TT	0.115	−1.453	0.464
			b*	GT	**0.001**	**−3.205**	**0.201**
				TT	**2.134 × 10^{-04}**	**−4.725**	**0.617**
			ΔE	GT	0.691	−0.118	0.439
				TT	0.039	−0.803	0.039
SLC45A2	rs16891982	GG	L*	GC	0.687	−0.327	0.176
				CC	0.876	−0.458	0.000
			a*	GC	**2.278 × 10^{-04}**	**3.675**	**0.340**
				CC	0.258	4.105	0.001
			b*	GC	**9.493 × 10^{-05}**	**5.458**	**0.968**
				CC	0.602	2.625	0.017
			ΔE	GC	0.647	−0.197	0.946
				CC	0.542	−0.961	0.106
TYR	rs1393350	GG*	L*	GA	0.907	−0.062	0.005
				AA	**0.004**	**3.185**	**0.109**
			a*	GA	0.214	−0.828	0.192
				AA	0.086	−2.367	0.999
			b*	GA	0.034	−1.974	0.201
				AA	0.127	−2.914	0.876
			ΔE	GA	0.845	0.055	0.158
				AA	0.005	1.614	0.033
IRF4	rs12203592	CC*	L*	CT	0.332	0.575	0.862
				TT	0.027	3.220	0.688
			a*	CT	0.016	−1.745	0.561
				TT	**0.001**	**−5.809**	**0.617**

Table 3. (continued)

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
			b*	CT	0.024	−2.282	0.651
				TT	**5.917×10^{-05}**	**−9.916**	**0.997**
			ΔE	CT	0.058	0.588	0.951
				TT	0.628	−0.375	0.487
TYRP1	rs1408799	CC	L*	CT	0.839	−0.111	0.506
				TT	0.054	−1.629	0.363
			a*	CT	0.254	0.776	0.820
				TT	0.008	2.811	0.864
			b*	CT	0.289	1.011	0.892
				TT	0.106	2.372	0.405
			ΔE	CT	0.149	−0.415	0.613
				TT	0.124	−0.684	0.111
NPLOC4	rs9894429	CC*	L*	CT	0.124	−0.925	0.179
				TT	0.698	−0.287	0.291
			a*	CT	0.844	−0.147	0.795
				TT	0.081	−1.608	0.465
			b*	CT	0.637	−0.494	0.349
				TT	0.029	−2.808	0.539
			ΔE	CT	0.399	0.267	0.194
				TT	0.155	0.553	0.477
LYST	rs3768056	AA*	L*	AG	0.166	−0.755	0.909
				GG	0.589	0.655	0.006
			a*	AG	0.098	1.122	0.395
				GG	0.636	0.712	0.094
			b*	AG	0.061	1.773	0.631
				GG	0.985	0.040	0.071
			ΔE	AG	0.198	−0.367	0.328
				GG	0.718	−0.228	0.004
ASIP	rs6058017	AA	L*	AG	0.143	−0.923	0.298
				GG	0.964	0.093	0.404
			a*	AG	0.027	1.737	0.479
				GG	0.682	1.053	0.528
			b*	AG	0.007	2.942	0.589
				GG	0.491	2.444	0.277
			ΔE	AG	0.380	−0.290	0.020
				GG	0.024	2.444	0.560

We report the P-value of the association, the effect size (beta) and the P-value for Cochran's Q statistic for each additional copy of the minor allele relative to the reference genotype. After the Bonferroni correction for multiple comparisons, associations were significant if $P < 0.00454$. Reference genotypes marked with an asterisk (*) represent the homozygous ancestral state. Significant associations are bolded and italicized.

increases the L*, a* and b* coordinates. Neither marker showed any significant deviations from an additive mode of inheritance.

The only marker that was associated with ΔE in the European sample was *HERC2* rs12913832. This marker had an additive effect, with each copy of the ancestral allele decreasing the colour difference between the ciliary and pupillary zones (Table 3a). In the South Asian sample, both *HERC2* rs12913832 and *SLC45A2* rs1426654 were associated with ΔE. *HERC2* rs12913832 showed significant deviations from additivity, suggesting that it is best modelled using a dominant/recessive mode of inheritance. In contrast, *SLC45A2* did not show any deviation from additivity. After conditioning for the effects of *HERC2* rs12913832, *SLC45A2* rs1426654 was no longer associated with ΔE.

Discussion

In this study, we present an improved method for measuring iris colour in diverse populations and looked at the association between 14 SNPs purported to play a role in global pigmentation variation and eye colour in a sample of East Asian, European and South Asian ancestry. CIE 1976 L*a*b* (CIELAB) colour space is an ideal system for measuring iris colour. This colour space was designed to capture perceptible differences in colour variation, which is important when looking at visible human traits that are typically distinguished by colour difference (McLaren, 1976). Each unit change in CIELAB colour space is visible to at least 50% of observers (Kuehni and Marcus, 1979). CIELAB also characterizes colour across three coordinates that closely parallel visual

Table 4. (a) Results of the meta-analysis in the South Asian sample; (b) Results of the meta-analysis in the South Asian sample after conditioning for the effects of HERC2 rs12913832

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
(a)							
HERC2	rs12913832	AA*	L*	AG	**5.042×10^{-14}**	**4.652**	**0.530**
				GG	**7.535×10^{-26}**	**17.867**	**0.879**
			a*	AG	**7.571×10^{-04}**	**1.601**	**0.885**
				GG	**9.703×10^{-17}**	**−10.856**	**0.897**
			b*	AG	**7.073×10^{-11}**	**4.165**	**0.669**
				GG	0.390	1.510	0.770
			ΔE	AG	**8.583×10^{-10}**	**1.305**	**0.074**
				GG	**6.443×10^{-38}**	**7.520**	**0.916**
SLC45A2	rs1426654	AA	L*	AG	**8.901×10^{-04}**	**−2.337**	**0.948**
				GG	**0.002**	**−3.673**	**0.689**
			a*	AG	**1.400×10^{-05}**	**−2.118**	**0.710**
				GG	**1.538×10^{-06}**	**−3.905**	**0.956**
			b*	AG	**6.703×10^{-09}**	**−3.512**	**0.789**
				GG	**5.271×10^{-09}**	**−5.881**	**0.921**
			ΔE	AG	**0.002**	**−0.775**	**0.527**
				GG	0.261	−0.476	0.379
SLC24A4	rs12896399	GG*	L*	GT	0.807	0.147	0.414
				TT	0.793	−0.299	0.871
			a*	GT	0.147	−0.615	0.133
				TT	0.343	−0.763	0.186
			b*	GT	0.172	−0.741	0.200
				TT	0.523	−0.657	0.470
			ΔE	GT	0.325	0.210	0.874
				TT	0.210	0.507	0.542
SLC45A2	rs16891982	CC*	L*	CG	0.943	−0.050	0.094
				GG	0.607	−1.597	0.427
			a*	CG	0.409	−0.408	0.225
				GG	0.701	0.849	0.431
			b*	CG	0.964	−0.028	0.114
				GG	0.398	2.385	0.365
			ΔE	CG	0.609	0.127	0.759
				GG	0.964	−0.050	0.639
TYR	rs1393350	GG*	L*	GA	0.534	0.510	0.321
				AA	0.907	−0.280	0.241
			a*	GA	0.715	−0.213	0.807
				AA	0.437	1.315	0.121
			b*	GA	0.752	0.233	0.135
				AA	0.572	1.217	0.170
			ΔE	GA	0.448	0.222	0.174
				AA	0.317	−0.850	0.962
TYRP1	rs1408799	TT*	L*	TC	0.116	0.946	0.170
				CC	0.795	0.252	0.857
			a*	TC	0.009	1.106	0.226
				CC	0.014	1.674	0.280
			b*	TC	0.024	1.224	0.606
				CC	0.007	2.366	0.671
			ΔE	TC	0.109	−0.344	0.027
				CC	0.509	−0.229	0.940
NPLOC4	rs9894429	TT	L*	TC	0.615	−0.315	0.382
				CC	0.802	−0.213	0.891
			a*	TC	0.297	−0.464	0.412
				CC	0.636	−0.284	0.877
			b*	TC	0.148	−0.819	0.696
				CC	0.163	−1.068	0.717
			ΔE	TC	0.198	0.283	0.097
				CC	0.839	−0.061	0.359

Table 4. (continued)

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
LYST	rs3768056	AA*	L*	AG	0.091	1.012	0.933
				GG	0.292	1.145	0.603
			a*	AG	**0.002**	**1.308**	**0.475**
				GG	0.046	1.544	0.168
			b*	AG	**0.002**	**1.696**	**0.808**
				GG	0.064	1.836	0.241
			ΔE	AG	0.730	−0.074	0.655
				GG	0.331	−0.380	0.630
DSCR9	rs7277820	GG*	L*	GA	0.961	−0.031	0.844
				AA	0.628	−0.450	0.174
			a*	GA	0.321	0.441	0.228
				AA	0.276	−0.714	0.114
			b*	GA	0.356	0.524	0.271
				AA	0.507	−0.556	0.050
			ΔE	GA	0.311	−0.228	0.907
				AA	0.556	−0.195	0.988
ASIP	rs6058017	AA	L*	AG	0.204	0.797	0.895
				GG	0.763	−0.356	0.655
			a*	AG	0.472	0.318	0.027
				GG	0.661	−0.367	0.523
			b*	AG	0.248	0.655	0.171
				GG	0.733	0.366	0.505
			ΔE	AG	0.406	0.185	0.168
				GG	0.750	0.134	0.349
(b)							
SLC45A2	rs1426654	AA	L*	AG	**0.003**	**−1.918**	**0.979**
				GG	**0.002**	**−3.255**	**0.637**
			a*	AG	**1.651×10^{-08}**	**−2.461**	**0.759**
				GG	**4.9×10^{-09}**	**−4.233**	**0.997**
			b*	AG	**5.237×10^{-09}**	**−3.560**	**0.823**
				GG	**4.738×10^{-09}**	**−5.922**	**0.902**
			ΔE	AG	0.007	−0.586	0.499
				GG	0.417	−0.292	0.283
SLC24A4	rs12896399	GG*	L*	GT	0.896	0.071	0.093
				TT	0.450	−0.778	0.329
			a*	GT	0.157	−0.554	0.337
				TT	0.547	−0.447	0.471
			b*	GT	0.167	−0.757	0.196
				TT	0.501	−0.699	0.461
			ΔE	GT	0.362	0.166	0.261
				TT	0.399	0.291	0.782
SLC45A2	rs16891982	CC*	L*	CG	0.386	−0.542	0.019
				GG	0.633	−1.320	0.381
			a*	CG	0.766	−0.135	0.328
				GG	0.744	0.662	0.389
			b*	CG	0.903	−0.078	0.097
				GG	0.404	2.365	0.370
			ΔE	CG	0.781	−0.059	0.357
				GG	0.957	0.051	0.572
TYR	rs1393350	GG*	L*	GA	0.492	0.507	0.568
				AA	0.965	0.094	0.198
			a*	GA	0.653	−0.240	0.474
				AA	0.494	1.059	0.089
			b*	GA	0.788	0.200	0.151
				AA	0.577	1.204	0.174
			ΔE	GA	0.358	0.228	0.347
				AA	0.331	−0.701	0.939

Table 4. (continued)

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
TYRP1	rs1408799	TT*	L*	TC	0.025	1.215	0.382
				CC	0.407	0.719	0.729
			a*	TC	0.021	0.905	0.405
				CC	0.032	1.343	0.269
			b*	TC	0.028	1.208	0.680
				CC	0.007	2.362	0.643
			ΔE	TC	0.225	−0.224	0.069
				CC	0.926	−0.027	0.945
NPLOC4	rs9894429	TT	L*	TC	0.601	−0.296	0.682
				CC	0.864	−0.130	0.751
			a*	TC	0.290	−0.432	0.630
				CC	0.532	−0.343	0.986
			b*	TC	0.177	−0.771	0.623
				CC	0.165	−1.066	0.715
			ΔE	TC	0.135	0.280	0.205
				CC	0.930	−0.022	0.383
LYST	rs3768056	AA*	L*	AG	0.041	1.093	0.828
				GG	0.369	0.870	0.231
			a*	AG	0.001	1.299	0.319
				GG	0.014	1.732	0.325
			b*	AG	0.002	1.744	0.743
				GG	0.069	1.808	0.227
			ΔE	AG	0.813	−0.042	0.713
				GG	0.137	−0.486	0.848
DSCR9	rs7277820	GG*	L*	GA	0.948	−0.037	0.658
				AA	0.264	−0.932	0.062
			a*	GA	0.287	0.437	0.484
				AA	0.485	−0.421	0.173
			b*	GA	0.378	0.506	0.273
				AA	0.469	−0.611	0.056
			ΔE	GA	0.204	−0.242	0.337
				AA	0.144	−0.410	0.635
ASIP	rs6058017	AA	L*	AG	0.168	0.778	0.307
				GG	0.429	−0.840	0.882
			a*	AG	0.476	0.291	0.089
				GG	0.884	−0.112	0.871
			b*	AG	0.278	0.621	0.145
				GG	0.790	0.288	0.549
			ΔE	AG	0.315	0.191	0.580
				GG	0.885	−0.052	0.759

We report the P-value of the association, the effect size (beta) and the P-value for Cochran's Q statistic for each additional copy of the minor allele relative to the reference genotype. After the Bonferroni correction for multiple comparisons, associations were significant if $P < 0.00500$. Reference genotypes marked with an asterisk (*) represent the homozygous ancestral state. Significant associations are bolded and italicized.

differences in eye colour. The L* dimension represents a brightness dimension and ranges from 0 to 100, with 0 being black and 100 being white. The a* and b* dimensions represent variation in colour, with negative values of a* indicating green and positive values of a* indicating red, and negative values of b* indicating blue and positive values of b* indicating yellow. CIELAB also has a colour metric, ΔE that can be used to quantify the difference between two points in colour space (McLaren, 1976). This allows us to investigate colour variation between different regions of the iris. Unlike previous studies, we selected a wedge to represent iris colour instead of the entire iris. We made this decision because the left quadrant of the iris was least likely to be obstructed in our sample. In addition, if we chose to use the entire iris but crop out regions of obstruction, such as eyelashes and eyelids, it would bias the colour of the iris towards the pupillary region. Although several automated methods have been developed to facilitate the isolation of the iris from photographs of the eye (Liu et al., 2010; Pietroni et al., 2014), we chose to manually define the boundaries of the iris. This allowed us to separate the eye into different regions and look at the difference in colour between the ciliary and pupillary zones.

Contrary to previous studies, we did not find any association between iris colour and age or sex in the East

Table 5. Results of the meta-analysis in the East Asian sample (a) before (b) after conditioning for the effects of *OCA2* rs1800414

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
(a)							
OCA2	rs1800414	GG	L*	GA	***1.434 × 10^{-05}***	***−1.460***	***0.503***
				AA	***5.875 × 10^{-09}***	***−2.793***	***0.902***
			a*	GA	***5.187 × 10^{-08}***	***−1.319***	***0.493***
				AA	***1.704 × 10^{-16}***	***−2.831***	***0.926***
			b*	GA	***1.137 × 10^{-07}***	***−1.757***	***0.510***
				AA	***1.308 × 10^{-17}***	***−4.024***	***0.783***
OCA2	rs74653330	GG*	L*	GA	0.069	1.201	0.177
				AA	–	–	–
			a*	GA	***4.310 × 10^{-04}***	***1.700***	***0.153***
				AA	–	–	–
			b*	GA	***1.141 × 10^{-04}***	***2.556***	***0.063***
				AA	–	–	–
SLC24A4	rs12896399	GG*	L*	GT	0.273	−0.375	0.432
				TT	0.902	−0.067	0.267
			a*	GT	0.088	−0.429	0.263
				TT	0.161	−0.562	0.190
			b*	GT	0.027	−0.761	0.129
				TT	0.018	−1.294	0.057
DSCR9	rs7277820	GG*	L*	GA	0.186	0.477	0.296
				AA	0.251	0.521	0.499
			a*	GA	0.140	0.395	0.984
				AA	0.087	0.577	0.468
			b*	GA	0.042	0.745	0.734
				AA	0.024	1.041	0.609
NPLOC4	rs9894429	TT	L*	TC	0.634	−0.161	0.506
				CC	0.844	0.170	0.089
			a*	TC	0.782	−0.069	0.648
				CC	0.990	0.008	0.154
			b*	TC	0.442	−0.265	0.739
				CC	0.590	−0.474	0.337
LYST	rs3768056	AA*	L*	AG	0.306	0.359	0.323
				GG	0.379	0.879	0.001
			a*	AG	0.168	0.364	0.471
				GG	0.703	0.288	0.748
			b*	AG	0.161	0.505	0.388
				GG	0.397	0.873	0.032
ASIP	rs6058017	AA	L*	AG	0.338	0.332	0.796
				GG	0.434	0.582	0.081
			a*	AG	0.670	0.109	0.754
				GG	0.725	0.195	0.093
			b*	AG	0.921	0.035	0.682
				GG	0.707	0.283	0.003
(b)							
OCA2	rs1800414	GG	L*	GA	–	–	–
				AA	–	–	–
			a*	GA	–	–	–
				AA	–	–	–
			b*	GA	–	–	–
				AA	–	–	–
OCA2	rs74653330	GG*	L*	GA	***8.781 × 10^{-04}***	***2.151***	***0.298***
				AA	–	–	–
			a*	GA	***3.071 × 10^{-09}***	***2.671***	***0.310***
				AA	–	–	–
			b*	GA	***1.847 × 10^{-10}***	***3.918***	***0.143***
				AA	–	–	–
SLC24A4	rs12896399	GG*	L*	GT	0.304	−0.347	0.315
				TT	0.984	−0.011	0.373
			a*	GT	0.085	−0.420	0.138

Table 5. (continued)

Gene	Marker	Reference genotype	Colour metric	Genotype	Significance (P)	Beta	Heterogeneity (P)
				TT	0.189	−0.509	0.229
			b*	GT	0.023	−0.754	0.052
				TT	0.021	−1.213	0.112
DSCR9	rs7277820	GG*	L*	GA	0.275	0.388	0.321
				AA	0.495	0.306	0.468
			a*	GA	0.255	0.294	0.888
				AA	0.282	0.352	0.416
			b*	GA	0.090	0.599	0.808
				AA	0.107	0.718	0.567
NPLOC4	rs9894429	TT	L*	TC	0.712	−0.123	0.274
				CC	0.796	0.220	0.132
			a*	TC	0.812	−0.058	0.259
				CC	0.908	0.071	0.255
			b*	TC	0.451	−0.250	0.726
				CC	0.645	−0.388	0.525
LYST	rs3768056	AA*	L*	AG	0.293	0.363	0.298
				GG	0.445	0.749	0.002
			a*	AG	0.146	0.369	0.410
				GG	0.819	0.167	0.814
			b*	AG	0.138	0.510	0.324
				GG	0.474	0.706	0.032
ASIP	rs6058017	AA	L*	AG	0.491	0.235	0.802
				GG	0.480	0.519	0.117
			a*	AG	0.971	0.009	0.751
				GG	0.816	0.124	0.157
			b*	AG	0.744	−0.110	0.681
				GG	0.805	0.178	0.006

We report the P-value of the association, the effect size (beta) and the P-value for Cochran's Q statistic for each additional copy of the minor allele relative to the reference genotype. After the Bonferroni correction for multiple comparisons, associations were significant if $P < 0.00714$. Reference genotypes marked with an asterisk (*) represent the homozygous ancestral state. Significant associations are bolded and italicized.

Asian, European, or South Asian sample populations. Iris colour has been tentatively associated with sex in a small number of European populations (Pietroni et al., 2014). It has been suggested that this association may be highly population specific. As we looked at a broad range of biogeographical ancestries across Europe and had a small number of male participants, it is not surprising that this association was absent in our sample. Similarly, the lack of an association between age and eye colour is not surprising, as we sampled from a relatively narrow age range compared to other studies that included participants that ranged from children to seniors (Bito et al., 1997).

Iris colour in European populations

The European sample showed the greatest variation and spread across CIELAB colour space. This is to be expected, given that eye colour in this population ranges from blues, to greens to browns. Interestingly, very few individuals of European descent fell into the region of the colour space occupied by the darkest brown irises. Instead, when individuals of European ancestry had brown eyes, they tended to be lighter. This suggests that there may be fixed genetic markers in this population that modulate the intensity of brown iris pigmentation.

In recent years, a number of iris colour prediction models have been developed by forensic groups (Ruiz et al., 2014; Spichenok et al., 2011; Walsh et al., 2011, 2013). These models predict the iris colour of an individual based on their genotype at a set of established iris pigmentation markers. The best known of these is perhaps IrisPlex, a forensically validated genetic assay and prediction model that uses 6 iris colour polymorphisms (*HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs129896399, *SLC45A2* rs16891982, *TYR* rs1393350, *IRF4* rs12203592) to estimate blue, brown and intermediate eye colour from unknown DNA samples (Walsh et al., 2011, 2013). A second system suggested that the inclusion of additional markers from the *HERC2-OCA2* region could improve the prediction of intermediate iris colour phenotypes (Ruiz et al., 2014). A third system, known as 7-Plex was designed to estimate both skin and iris colour (Spichenok et al., 2011). This system uses three SNPs (*HERC2* rs12913832, *SLC45A2* rs16891982, *IRF4* rs12203592) associated with iris and skin colour, three SNPs associated with genetic ancestry (*MC1R* rs885479, *ASIP* rs6119471 and *OCA2* rs1545397) and

one SNP associated with skin colour alone (*SLC24A5* rs1426654) to predict brown, not brown, not blue and green eye colour. Apart from the markers that are currently incorporated into iris prediction models, several studies have identified additional polymorphisms that may have an effect on iris colour. *TYRP1* rs1408799 has been associated with the difference between 'blue and not blue' eyes in a Northern European population and *ASIP* rs6058017 may play some role in modulating brown iris colour (Kanetsky et al., 2002; Sulem et al., 2008). In addition, three new potential iris colour predictors (*DSCR9* rs7277820, *NPLOC4* rs9894429, *LYST* rs3768056) were recently identified in a genomewide association study (Liu et al., 2010). However, as of yet, none of these markers have been incorporated into any forensic algorithms.

In our sample, *HERC2* rs1291832 had the strongest effect on eye colour across all three dimensions of colour space. *HERC2* rs12913832 has been traditionally characterized as having a dominant/recessive mode of inheritance, where heterozygotes and homozygotes for the ancestral allele have brown or intermediate eyes and homozygotes for the derived allele have blue eyes. This was largely reflected in our sample, as both the a* and b* dimensions of colour space showed significant deviations from additivity. In contrast, the effect of *HERC2* rs12913832 on the L* coordinate was largely additive, with one copy of the ancestral allele increasing the brightness in the eye by 7.143 units and two copies increasing it by 12.586 units. This suggests that the inheritance of this polymorphism in European populations is more complex than traditionally modelled. Although this marker may control the difference between blue and brown eye colour, it also has more subtle effects on the overall lightness of the iris. The additive nature of *HERC2* rs12913832 is supported by recent functional studies (Cook et al., 2009). Cultured melanocyte strains that were homozygous for the ancestral allele had more melanin content than homozygotes for the derived allele, while heterozygotes had intermediate amounts. Thus, it appears that *HERC2* rs12913832 may have both dominant and additive effects on iris colour variation, depending on which dimensions of colour space are being studied.

In addition to *HERC2* rs12913832, our study supports previous research showing that *OCA2* rs1800407, *SLC45A2* rs16891982, *SLC24A4* rs12896399, *IRF4* rs12203592 and *TYR* rs1393350 are the primary determinants of iris colour in European populations (Eiberg et al., 2008; Kayser et al., 2008; Liu et al., 2010; Sturm et al., 2008; Walsh et al., 2011). All of these markers were significantly associated with iris colour in at least one dimension either before or after conditioning for *HERC2* rs12913832. We were not able to identify an association between iris colour variation and any of the other markers. However, after conditioning for *HERC2* rs12913832, having two copies of the derived allele at *TYRP1* rs1408799 showed a borderline significant association with the L* ($P = 0.054$, $\beta = -1.629$) and a* ($P = 0.007$, $\beta = 3.091$) dimensions of colour space and having one copy of the ancestral allele at ASIP rs6058017 showed a borderline significant association ($P = 0.007$, $\beta = 2.942$) with the b* dimension. It is possible that our sample size was too small to pick up these associations as significant.

Iris colour in South Asian populations

The South Asian sample showed considerably less diversity across CIELAB colour space than the European sample, and the vast majority of participants in this sample had eyes that would be traditionally described as brown. However, there were still a small number of participants that fell into the intermediate region of colour space.

At present, very little research has been devoted to the study of iris colour in populations of South Asian ancestry. *HERC2* rs12913832 is present at low frequencies in South Asia (Edwards et al., 2012). In this population, this polymorphism appears to modulate variation in brown iris colour. In a recent study on brown-eyed individuals of South Asian ancestry, one copy of the derived G allele was found to significantly increase the L*, a* and b* dimensions of CIELAB colour space (Edwards et al., 2012). However, there was only one homozygote for the derived allele in that study. Apart from *HERC2* rs12913832, the role that other iris colour markers play in South Asia has not yet been tested. Both *OCA2* rs1800407 and *IRF4* rs12203592 have very low minor allele frequencies (MAF) in this population and are unlikely to contribute to normal pigmentation variation.

In our South Asian sample, we were able to replicate the association between *HERC2* rs12913832 and iris colour variation. This marker showed significant deviations from additivity across all three dimensions of colour space. Having two copies of the derived allele increased L* values by 17.87 and decreased a* values by 10.8562 relative to the ancestral homozygotes. These effects are similar to what was observed in the European sample. However, there is a clear difference in the effect of *HERC2* rs12913832 in the b* dimension of colour space in Europeans and South Asians. In Europeans, the derived homozygote is associated with a very strong reduction in the b* values with respect to the ancestral homozygote (~20 units). In contrast, in the South Asian sample, the derived homozygote has no significant effect on b* values. In fact, in this sample, derived homozygotes have slightly higher b* values than the ancestral homozygote. In our South Asian sample, there were only 7 individuals homozygous for the derived *HERC2* rs12913832 allele. These individuals had an average b* value between 8 and 9 (depending on the camera), indicative of intermediate colour irises, in contrast to the average b* values observed in individuals homozygous for the derived allele in Europe, which is around −7, within the blue region of

the colour space. Additionally, the South Asian individuals homozygous for the ancestral allele have much lower b* values (average 7–8, depending on the camera) than the European ancestral homozygotes (average 13–15, depending on the camera). This is primarily due to the fact that the South Asian individuals have darker brown irises than the Europeans, and dark brown colours have lower b* values than light brown colours. The difference in the effect of the *HERC2* rs12913832 polymorphism in Europe versus South Asia strongly suggests that there are other polymorphisms modifying the effect of this marker in both populations. Our hypothesis is that the effect of *HERC2* rs12913832 may be modified by other variants that are common in Europe, but not in South Asia. Exploring this would require to gather much larger samples including substantial numbers of derived homozygotes in South Asia, to explore potential interactions of *HERC2* rs12913832 with other pigmentation variants.

The only other marker that had a strong effect on iris pigmentation in the South Asian sample was *SLC45A2* rs1426654. *SLC45A2* rs1426654 is a non-synonymous polymorphism (A111T) that results in the substitution of a guanine to an adenine in exon three of *SLC45A2*. This marker shows strong signals of positive selection in Europe and South Asia and has been associated with skin pigmentation variation in South Asians and also in admixed individuals of African European descent (Lamason et al., 2005; Stokowski et al., 2007). Functional analyses have found that homozygotes for the ancestral G allele have 2.2-fold higher melanin content and 1.7 higher TYR activity than homozygotes for the derived A allele (Cook et al., 2009). This is not the first study to suggest that this marker may play some role in iris pigmentation variation. Beleza et al. (2013) found that this marker was strongly associated with iris colour in an admixed population of African European ancestry. In our study, *SLC45A2* rs1426654 was one of the main determinants of iris colour in South Asians. This marker showed an additive mode of inheritance for all three dimensions of colour space, with one copy of the ancestral allele decreasing the L*, a* and b* values by 2.337, 2.118 and 3.512, respectively, and a second copy decreasing the values by 3.673, 3.905 and 5.882 relative to the homozygous-derived genotype. This suggests that the ancestral G allele is responsible for darkening brown iris colour in this population. It is important to note that in our South Asian sample, *SLC45A2* rs1426654 showed significant deviations from Hardy–Weinberg proportions. This is not surprising as there is well-documented population stratification in South Asia and this marker is under strong global selection (Izagirre et al., 2006; McEvoy et al., 2006; Stokowski et al., 2007). Although it is possible that the association between this marker and iris colour is a secondary effect resulting from population stratification, it is not likely. *SLC24A4* rs1426654 has well-established functional effects on the synthesis of melanin and was the only marker that showed major deviations from Hardy–Weinberg proportions in this group.

Although *SLC45A2* rs1426654 and *HERC2* rs12913832 were the primary determinants of iris colour in the South Asian sample, *LYST* rs3768056 also showed a much smaller association with the a* and b* dimensions of CIELAB colour space, with the derived G allele increasing both coordinates. The association between *LYST* rs3768056 and iris pigmentation in the South Asian population is interesting, as this is one of the new putative pigmentation markers that was suggested for the European population (Liu et al., 2010). This marker may be a stronger predictor of iris colour in populations of non-European ancestry. In addition, having one copy of the derived T allele at *TYRP1* rs1408799 showed a borderline significant effect on the a* and b* dimensions of colour space, with the derived C allele increasing both coordinates.

Iris colour in East Asian populations

Iris colour showed the most limited distribution in the East Asian sample. Blue iris colour was completely absent in this group, and only a small number of eyes had an intermediate or green phenotype. The majority of irises fell into the region of colour space associated with various shades of brown. Average iris colour was comparable to the South Asian sample.

Very little is known about pigmentation phenotypes in East Asia. Light skin pigmentation appears to have evolved independently Europe and East Asia, and there is very little overlap in the markers responsible for pigmentation diversity between these two populations (Eaton et al., 2015; Edwards et al., 2010; McEvoy et al., 2006; Norton et al., 2007). The majority of polymorphisms associated with light skin pigmentation in Europe and South Asia are absent in East Asia. In addition, the only putative iris pigmentation markers that have a MAF allele frequency greater than 0.05 are *SLC24A4* rs129896399, *DSCR9* rs7277820, *NPLOC4* rs9894429, *LYST* 3768056 and *ASIP* rs6058017. Thus, the genetic basis of iris pigmentation in East Asian populations is likely very different than in other groups.

The strongest determinants of iris pigmentation diversity in our sample were *OCA2* rs1800414 and *OCA2* rs74653330. *OCA2* rs1800414 had an additive effect on all three dimensions of CIELAB colour space. One copy of the ancestral A allele decreased the value of L*, a* and b* by 1.460, 1.318 and 1.757, respectively, and two copies of the ancestral A allele decreased the value by 2.793, 2.831 and 4.024. This suggests that the ancestral allele for this marker plays a role in darkening iris colour. Although we did not have any participants who were homozygous for the derived A allele for *OCA2* rs74653330, having one copy of the derived allele increased both the a* and b* values. After conditioning for *OCA2* rs1800414, the effect of *OCA2* rs74653330 became even stronger, with heterozygotes having L*, a*

and b* values that were 2.1509, 2.6713, 3.9180 higher respectively than the homozygous ancestral genotype.

OCA2 rs1800414 is a non-synonymous polymorphism (His615Arg) that is present in very high frequencies in East Asia (Donnelly et al., 2012; Yuasa et al., 2007). The derived 'G' allele is strongly associated with lower skin melanin levels, with each copy of the G allele decreasing the skin melanin index by approximately 0.9 units (Edwards et al., 2010). *OCA2* rs74653330 is another non-synonymous (Ala481Thr) polymorphism that is largely restricted to East Asia (Eaton et al., 2015). The derived T allele has a low frequency in this region. Each copy of the derived T allele has been found to reduce the skin melanin index by approximately 1.9 units (Eaton et al., 2015). The *OCA2* gene appears to be under strong selective pressure in East Asia (Donnelly et al., 2012; Eaton et al., 2015; Edwards et al., 2010; Lao et al., 2007). Given the role that the derived allele plays in lightening skin pigmentation for both of these markers, it is likely that their influence on iris colour is only secondary.

Central heterochromia

Central heterochromia is a trait that is prevalent in populations with lighter coloured irises (Larsson et al., 2011). It usually takes the form of a blue/green iris with a ring of darker pigment around the pupil. When central heterochromia is present in the iris, darker colour is typically restricted to the pupillary zone of the iris, although it occasionally extends into the ciliary zone. To characterize the magnitude of central heterochromia in the iris, we looked at the difference in iris colour between the ciliary and pupillary zones. We used the ΔE metric to quantify this trait, as it was designed to capture perceptible differences in colour across CIELAB colour space (Kuehni and Marcus, 1979).

In our sample, the degree of central heterochromia was largely population dependent, with the European group showing the highest prevalence of this trait (Table 2). There was some heterochromia in the South Asian sample; however, most irises had very low ΔE values. The East Asian group showed the least colour difference. As heterochromia was largely absent in our East Asian sample, we only looked at the association between the putative pigmentation markers and heterochromia in the European and South Asian samples.

The only polymorphism that was associated with central heterochromia in the European sample was *HERC2* rs12913832. The effect of this marker appeared to be additive, with one copy of the ancestral allele decreasing the colour difference by 1.220, and two copies of the ancestral allele decreasing the colour difference by 3.828 relative to the homozygous-derived genotype. *HERC2* rs12913832 was also associated with central heterochromia in the South Asian population. However, in this group, it appears to be better modelled using a dominant/recessive mode of inheritance, with one copy of the derived allele increasing the colour difference by 1.305 and two copies increasing the colour difference by 7.520 relative to the homozygous ancestral genotype. It is interesting to note that heterozygotes for this marker in the European and South Asian population show very different amounts of colour difference (Table 6). In the European sample, the average ΔE ranged between 6.668 and 8.128 when *HERC2* rs12913832 is in the heterozygous state. In the South Asian sample, however, the average ΔE only ranged between 3.634 and 3.722. In contrast, when this marker is found in the derived homozygous state, both groups showed closer amounts of colour difference. It is well known that *HERC2* rs12913832 can have an effect on intermediate phenotypes. (Spichenok et al., 2011; Walsh et al., 2011, 2012). In particular, individuals who are heterozygous for this marker commonly have iris colours that range from blues, to greens to browns. However, it is likely that there are other polymorphisms interacting with *HERC2* rs12913832 to produce variation in central heterochromia. This is particularly evident when looking at the average ΔE in heterozygotes for this marker in the European and South Asian groups. Although heterozygotes in the European sample show substantial amounts of central heterochromia, this is not the case in the South Asian sample. Therefore, there are likely other polymorphisms modulating the effect of this marker in both of these groups.

Larsson et al. (2011) identified an association between *SLC24A4* rs12896399 and heterochromatic variation in a genomewide association study performed in a sample of European ancestry living in Australia. However, we were not able to replicate this finding in our study. This may be because they looked at both the extent and spread of central heterochromia, and we only looked at colour difference. Interestingly, we did find a borderline significant effect between ΔE and *TYR* rs1393350 after conditioning for *HERC2* rs12913832 in the European sample, with the derived A allele increasing the amount of heterochromia in the eye. *TYR* rs1393350 has been previously associated with the difference between blue and green eyes (Sulem et al., 2008). Likewise, in the South Asian sample, *SLC45A2* rs1526654 was associated with ΔE before conditioning for *HERC2* rs12913832. However, after conditioning, this marker was no longer significant.

There are a number of structural differences between the pupillary and ciliary zones, including the thickness and opacity of these regions (MacKey et al., 2011; Oyster, 1999). It is possible that markers associated with the structure of the iris may also be determinants of central heterochromia.

A global view of the genetic basis of iris pigmentation

Although the evolution and genetic basis of iris colour in European populations has been well-studied over the past decade, very few research groups have attempted

Table 6. The average, minimum and maximum ΔE stratified by HERC2 rs12913832 genotype

	HERC2 Genotype	Camera	Average ΔE	Minimum ΔE	Maximum ΔE
European	AA*	Camera 1	4.806	0.823	11.503
		Camera 2	4.578	0.509	9.936
	AG	Camera 1	8.128	1.394	16.527
		Camera 2	6.668	0.683	16.153
	GG	Camera 1	9.669	1.515	19.264
		Camera 2	7.751	1.605	16.921
South Asian	AA*	Camera 1	2.589	0.247	9.433
		Camera 2	2.015	0.156	5.655
	AG	Camera 1	3.634	0.957	10.391
		Camera 2	3.722	0.511	9.918
	GG	Camera 1	10.043	5.328	13.486
		Camera 2	9.598	7.545	11.616

We report the average, minimum and maximum ΔE values for the three possible *HERC2* rs12913832 genotypes for both the European and South Asian populations. A* is used to represent the homozygous ancestral state. The East Asian sample was monomorphic for this marker, and was not included in the table. Note the substantial difference in average ΔE between the heterozygous European and South Asian samples.

to explore the genetic basis of iris pigmentation variation in populations of non-European ancestry. Although European populations may show the greatest diversity in eye colour phenotypes, iris colour variation extends far past the difference between blue and brown. This is especially evident when looking at the distribution of iris pigmentation in East and South Asian samples across CIE 1976 L*a*b (CIELAB) colour space. Although the majority of the irises in these populations would traditionally be described as brown, these browns showed much diversity and ranged from very light to very dark in colour. Thus, studying iris colour in populations with more homogenously coloured irises will allow us to approach the study of iris colour from a number of new directions and better understand the global diversity in this trait.

It is interesting to note that the markers associated with iris colour variation in all three groups have largely been associated with other pigmentary traits. *SLC45A2* rs16891982, *SLC24A5* rs1426654, *OCA2* rs1800414 and *OCA2* rs7465330 are responsible for major differences in global skin pigmentation diversity (Eaton et al., 2015; Edwards et al., 2010; Graf et al., 2005; Lamason et al., 2005; Stokowski et al., 2007). In addition, *SLC24A4* rs12896399 and *HERC2* rs12913832 have been associated with hair colour variation and *IRF4* rs12203592 has been associated with both hair and skin pigmentation (Han et al., 2008; Sulem et al., 2007). *TYR* rs1126809, which is in strong linkage disequilibrium with *TYR* rs1393350, has also been associated with both skin and hair colour (Nan et al., 2009; Sulem et al., 2008). Thus, it is very likely that many of the iris pigmentation markers were selected for because of the effect that they had on other pigmentary characteristics and not for their effect on iris colour variation. *HERC2* rs12913832, which shows evidence of strong positive selection in regions where blue eyes dominate, may be the exception (Donnelly et al., 2012).

It is also interesting to note that the markers which modulate iris colour variation in each region appear to be very different. The six markers commonly used as predictors in European populations were all significant in our European sample. However, only *HERC2* rs12913832 was associated with eye colour in the South Asian sample and none of the markers were associated with eye colour in East Asia. Rather, the markers that were significantly associated with iris colour variation in the East and South Asian populations were largely polymorphisms that played a role in skin and hair pigmentation variation in those regions. These include *SLC24A5* rs1426654 in the South Asian sample and *OCA2* rs1800414 and *OCA2* rs7465330 in the East Asian sample.

Continuing to develop a better understanding of the global distribution of iris colour variation will have a number of advantages. At present, the markers associated with intermediate eye colours are largely unknown. As a result, forensic eye colour predictor models have been found to perform poorly when applied to populations with a large proportion of green or intermediate eyes (Dembinski and Picard, 2014; Pneuman et al., 2012; Yun et al., 2014). As the genetic basis of iris colour appears to be highly population specific, it is critical to study eye colour in populations outside of Europe to get a better understanding of the genetic architecture of this trait. Determining the variants responsible for variation in brown iris colour may allow future iris predictor systems to distinguish between dark and light brown eyes in populations with more homogenous irises. This would broaden their use beyond European populations. Lastly, given that the markers associated with iris pigmentation appear to be strongly associated with skin and hair pigmentation variation, identifying novel variants associated with iris colour variation in populations of non-European ancestry may provide valuable information about the genetic basis of skin and hair colour in these regions.

The development of quantitative methods of measuring iris colour has opened up many doors in pigmentation research. However, these methods have not yet been widely applied to populations of non-European ancestry. In this study, we present a new method to estimate iris colour and heterochromia based on high-resolution photographs. This method has been implemented in a Web application that can be accessed at http://iris.davidcha.ca/. Accounts can be set up for interested users by request. Using this quantitative approach, we identified one novel variant associated with iris colour in a South Asian sample and two novel variants in an East Asian sample. We suggest that future research should apply such quantitative methods to other global populations, such as African American and Hispanic groups. In addition, as the genetic basis of central heterochromia and intermediate irises continues to be poorly understood, we suggest that using quantitative methods that allow researchers to divide the iris into separate regions may provide a new approach for studying these traits.

Methods

Sample collection

Between 2012 and 2014, 1465 healthy volunteers of East Asian, European and South Asian ancestry volunteered for a research study on human pigmentation variation. All participants ranged between 18 and 35 yr of age and were recruited using online and print advertisements directed towards the University of Toronto student community. A personal questionnaire was administered to each participant to determine their age, sex, self-described eye colour and whether or not they had been diagnosed with any pigmentation-related diseases or disorders.

Biogeographical ancestry was determined using information from the personal questionnaire, which inquired about the ancestry, place of birth and first language of each participant's maternal and paternal grandparents. Individuals who stated that all of their grandparents originated in China, Japan, Korea or Taiwan were categorized as East Asian, and individuals who stated that all of their grandparents originated in Pakistan, India, Bangladesh or Sri Lanka were categorized as South Asian. Individuals were categorized as European if all of their grandparents originated in any country in Europe, other than Turkey. Admixed individuals who had grandparents from two different regions (i.e. East Asia and Europe) were excluded from the analysis. When information about the grandparents was not known, the self-described ancestry of both parents was used to assess biogeographical ancestry. In addition to the 1465 participants that were included in this paper, 308 additional volunteers were recruited and excluded because they could not be categorized as East Asian, European or South Asian using this criteria.

This study was approved by the University of Toronto Research and Ethics Board (Protocol Reference #27015), and all participants were required to provide written informed consent.

Acquisition of photographs

A photograph of each participant's right eye was taken using a Miles Research Professional Iris Camera (Miles Research, United States). This camera consists of a Fujifilm Finepix S3 Pro DSLR 12-megapixel camera body attached to a 105-mm Nikkor lens. A biometric coaxial cable was used to deliver light to the iris at a constant light temperature to maintain colour and brightness fidelity and reduce the impact of ambient light. The camera body needed to be replaced after the first 552 participants and a camera body with an identical make and model was acquired. We could not adequately assess if the photographs taken with the first and second camera bodies were identical as we did not have a large enough sample of individuals whose eyes had been photographed using both cameras. Therefore, we split our sample into two groups. All photographs were taken with an ISO of 200, a shutter speed of 1/125″ and an aperture of *f*19.

Photographs were initially acquired in RAW format and later converted to JPEG format using Adobe Camera Raw in Adobe Photoshop CS5 (Adobe Systems Incorporated, United States). They were resized from 3043 × 2036 pixels to 1200 × 803 pixels to optimize the processing of iris colour. The white balance was set to flash, the contrast and blacks levels were set to zero, and all other camera defaults were preserved for each conversion.

Acquisition of iris colour

One of the authors (D.C.) designed an eleven step Web-based application to characterize iris structure and acquire iris colour from photographs of the eye. The script used in the application is available in the supplementary file Data S1. The Web application can be accessed at http://iris.davidcha.ca/. Accounts can be set up for interested users by request. Only the first six steps are necessary to obtain a measurement of iris colour. In the first step, the user must note whether or not the iris is obstructed to an extent that could affect colour and structure measurements. In steps 2–6, the user is asked to identify the approximate centre point of the iris, the approximate centre point of the pupil and then draw a best fit circle around the scleral, pupillary and collarette boundaries.

After the scleral, pupillary and collarette boundaries are defined, the application is able to automatically extract a measurement of average eye colour. We decided to estimate iris colour from a 60° angle wedge taken from the left side of the iris. This region was chosen because it was least likely to be obscured by eyelids, eyelashes or reflections in our sample. To isolate this wedge, the program defines a start point located at the centre of the iris and moves to a point on the scleral boundary located 240° clockwise from the top of the iris. It then follows an arc that stretches for 60° until it reaches the point that is 300° from the top of the iris. This point is connected to the centre of the iris, which demarcates an isolated wedge. These steps are repeated for the pupillary boundary and the collarette boundary so that the pupil can be completely excised from the wedge and the pupillary and ciliary zones can be separated. Two images are then saved in PNG format: the portion of the wedge that represents the ciliary zone and the portion of the wedge that represents the pupillary zone (Figure 2). After processing all iris photographs, we manually scanned the wedges for any evidence of obstruction or incorrect cropping.

To obtain a measurement of average iris colour, the application counts all of the pixels located in the wedge (consisting of both the ciliary and pupillary zones) and determines a Red, Green and Blue (RGB) value for each individual pixel. The average RGB value of the entire wedge is calculated by adding up the R, G and B values for each pixel, and then dividing this value by the total number of counted pixels. We chose to describe iris colour in CIE 1976 L*a*b* (CIELAB) colour space (McLaren, 1976). CIELAB is a colour system that was designed to characterize colour across three different coordinates. The L* coordinate represents the lightness dimension and ranges from 0 to 100, with 0 being black and 100 being white. The a* and b* coordinates represent variation in colour, with negative values of a* indicating green and positive values of a* indicating red, and negative values of b* indicating blue and positive values of b* indicating yellow. To convert our RGB measurements into CIELAB colour space, the application first transformed the RGB coordinates into XYZ coordinates and then into L*, a* and b*

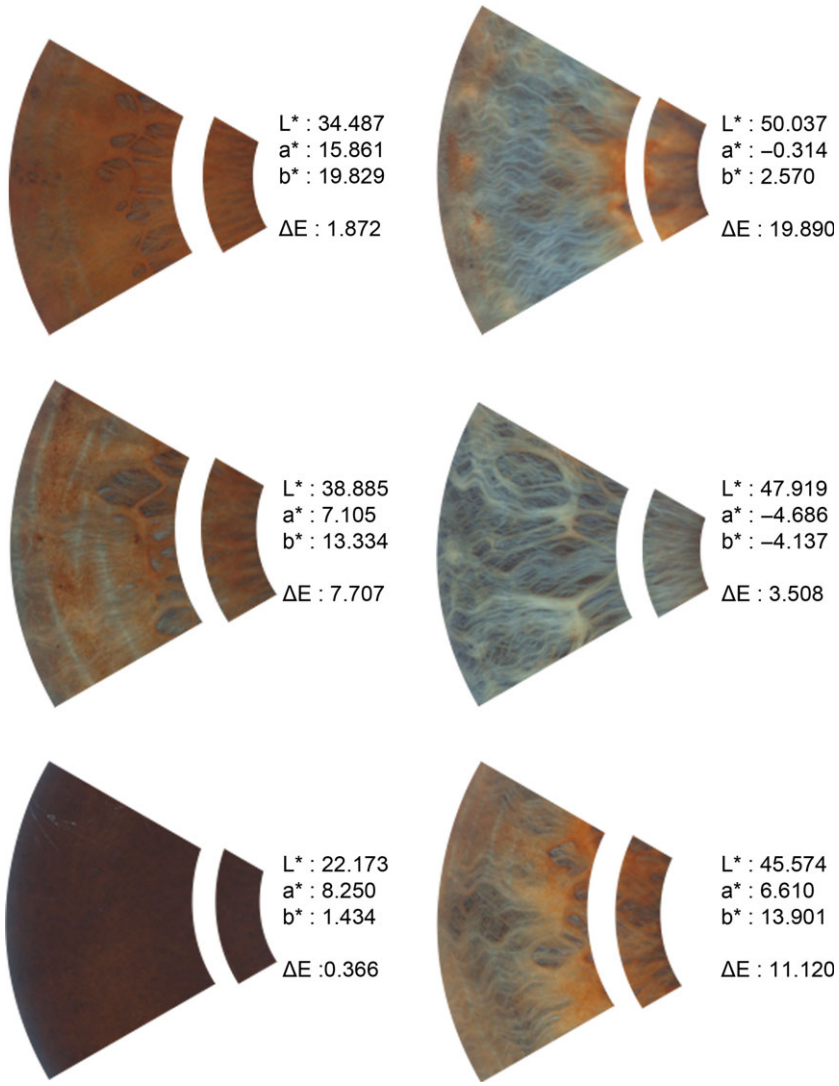

Figure 2. A wedge with a 60-degree angle is cropped from the left side of the iris based on the scleral and pupillary boundaries that the user manually defines during iris categorization. To obtain a measurement of average iris colour, the application determines the red, green and blue (RGB) value of each pixel located in the wedge and divides the sum of the RGB values by the total number of counted pixels. The RGB values are then transformed into CIE 1976 L*a*b* (CIELAB) colour space using an illuminant of D55 and an observer angle of 2 degrees. The Web application also divides the iris into a ciliary and pupillary zone based on the user-defined collarette. In the image above, we show the pupillary and ciliary wedges for six irises of differing colour. The L*, a*, b* and ΔE values are written to the right of each iris.

coordinates using the equations provided by EasyRGB (Logicol Color Technology, United States). For each conversion, the illuminant was set to D55 and the observer was set to 2 degrees. These are standard conversion settings when using flash photography. This process ultimately provides an average colour estimate for ciliary zone, the pupillary zone and the entire iris in CIELAB colour space.

We were also interested in quantifying the total amount of central heterochromia in the iris. To do this, we used CIEDE2000 (ΔE), a colour metric that looks at the difference between two colours in CIELAB colour space (McLaren, 1976). The Web application used the equations provided by EasyRGB (Logicol Color Technology, United States) to calculate the difference between the CIELAB values in the pupillary and ciliary zones.

After acquiring estimates of iris colour from all 1465 irises, the Web application was used to output an excel spreadsheet which contained the average colour of the entire iris, the average colour of the ciliary zone and the average colour of the pupillary zone in RGB and CIELAB colour space and the ΔE between the ciliary and pupillary zones. The spreadsheet also produced information on how many pixels were counted in each eye during the calculation of iris colour. All iris analyses were carried out by M.E.

Marker selection and genotyping

A 2-ml saliva sample was obtained from each participant using the Oragene•DNA (OG-500) collection kit (DNA Genotek, Canada). All participants were instructed not to eat, drink or smoke for at least 30 min prior to obtaining the sample to ensure maximal sample purity. DNA was isolated from each sample using the protocol provided by DNA Genotek and eluted in 500 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) Buffer.

We selected fourteen markers for genotyping that have either previously been associated with iris colour in European populations or been associated with some other pigmentation phenotype (i.e. skin colour) in East or South Asia (Eaton et al., 2015; Edwards et al., 2012; Eiberg et al., 2008; Graf et al., 2005; Kayser et al., 2008; Liu et al., 2010; Rebbeck et al., 2002; Sturm et al., 2008; Walsh et al., 2011). These markers consisted of *HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *SLC45A2* rs16891982, *SLC24A5* rs1426654, *TYR* rs1393350, *IRF4* rs12203592, *DSCR9* rs7277820, *TYRP1* rs1408799, *NPLOC4* rs9894429, *LYST* rs3768056, *ASIP* rs6058017, *OCA2* rs1800414 and *OCA2* rs74653330. Markers were only included for a population if the minor allele frequency (MAF) exceeded 0.05. The exception to this was *OCA2* rs74653330, which

has a low MAF (< 0.04) in East Asia, but has been found to have a strong effect on skin pigmentation variation (Eaton et al., 2015).

All DNA samples were sent to LGC Genomics (United States) for genotyping. LGC Genomics uses a KASP-based genotyping method that combines allele-specific amplification with FRET (fluorescent resonance energy transfer) technology. Twenty-nine samples were sent as blind duplicates and 14 samples were sent as blanks to validate the quality of the genotyping results. The concordance rate for both blind duplicates and blanks was 100%.

Statistical analysis

Unless otherwise noted, all statistical analyses were carried out using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/), a genome analysis program designed to look at the association between genotype and phenotype data. We carried out the following analyses: (i) A test of deviations from Hardy–Weinberg proportions for each marker included in the East Asian, European and South Asian sample. (ii) A linkage disequilibrium test to determine the amount of linkage between the markers. (iii) An exploratory analysis to look at the association between age and sex and the four colour space measurements (L*, a*, b* and ΔE). As no significant associations were noted between iris colour and age or sex, these variables were not included in the downstream statistical analyses. (iv) A linear regression analysis to determine the effect of each SNP on the L*, a* and b* dimensions of CIELAB colour space. In each population, we only looked at SNPs that reached a MAF of at least 0.05 (with the exception of *OCA2* rs74653330 in the East Asian sample). For each SNP, we used a genotypic model, which reports a P-value and beta coefficient independently for the minor allele homozygote and the heterozygote, with respect to the major allele homozygote. We also explored whether there was evidence for deviations from an additive model of inheritance for any of the markers. Deviations were considered significant if the statistic reporting deviation from additivity (DOMDEV) yielded a P-value of < 0.05 for both cameras. Each population was divided into two groups (representing the first camera body and the second camera body) and the regression was carried out individually on each group to account for differences in colour and brightness between the two cameras. (v) A linear regression analysis in the European and South Asian samples conditioning on the effect of *HERC2* rs12913832 and a linear regression analysis in the East Asian sample conditioning on the effect of *OCA2* 1800414. Again, this analysis was performed separately on each camera body. (vi) A meta-analysis of the linear regression results from both camera bodies. This meta-analysis was carried out both before and after conditioning on *HERC2* rs12913832 in the European and South Asian samples and *OCA2* rs1800414 in the East Asian sample. For each marker, we reported the P-value of the association, the effect size (beta) and the P-value for Cochran's Q statistic (a measure of the heterogeneity between the two groups used in the meta-analysis). After Bonferroni's correction for multiple comparisons, associations were significant in the East Asian population if $P < 0.00714$, in the European population if $P < 0.00454$ and in the South Asian population if $P < 0.00500$. (vii) A linear regression analysis in the European and South Asian samples looking at the association between the colour difference between the pupillary and ciliary zones (ΔE) and the putative pigmentation SNPs. This analysis was carried out separately on each camera body. (viii) A meta-analysis on linear regression results for ΔE from each camera body. The P-value of the association, the effect size (beta) and the P-value for Cochran's Q statistic were reported for each marker. (ix) Four months after the initial iris colour classification, intrarater reliability measurements were carried out by M.E and inter-rater reliability measurements were carried out by D.C on 40 random irises. The intraclass correlation coefficient between the original and repeated iris colour measurements was calculated in IBM Statistics SPSS (version 20.0, SPSS Incorporated, United States) using a two-way random model with absolute agreement for the L*, a*, b* and ΔE measurements.

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Supporting information

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

Figure S1. The distribution of irises across the a* and L* (A), b* and L* (B), and the a* and b* (C) coordinates of CIE 1976 L*a*b* (CIELAB) colour space for the first camera body.

Data S1. Script used in the iris colour web application.