UDC 575.
https://doi.org/10.2298/GENSR2302729V
Original scientific article

EVALUATION OF THE IRISPLEX SYSTEM FOR EYE COLOUR PREDICTION IN THE SERBIAN POPULATION

Marija VUKOVIC*, Milica MIHAJLOVIC SREJIC*, Katarina SAPIC, Vanja TANASIC, Verica RADOJICIC, Milica KECKAREVIC MARKOVIC, Miljana KECMANOVIC, Dusan KECKAREVIC

Centre for Forensic and Applied Molecular Genetics, Faculty of Biology, University of Belgrade, Belgrade, Serbia
*equally contributed

Vukovic M., M. Mihajlovic Srejic, K. Sapic, V. Tanasic, V. Radojicic, M. Keckarevic Markovic, M. Kecmanovic, D. Keckarevic (2023). *Evaluation of the Irisplex system for eye colour prediction in the Serbian population.* - Genetika, Vol 55, No.2,729 -741.

DNA-based prediction of a physical appearance, also known as DNA phenotyping, is a rapidly developing field with great potential for solving difficult forensic investigations. Externally visible characteristics (EVCs), such as eye colour, are easily recognised and genetically determined. Analysis of highly informative single nucleotide polymorphisms (SNPs) encompassing trait-associated genes provides information about the phenotype of an unknown individual, which is critical in cases where the standard STR profile is not useful. The IrisPlex assay was developed based on genotype data from 3804 Dutch Europeans, with the goal of accurately predicting brown, blue, and undefined eye colours using the six SNP markers alone. This assay has been validated in several studies and has shown high accuracy of prediction for brown and blue eye colours in most European populations. The aim of this work was to evaluate IrisPlex in the Serbian population and to determine if there are possible discrepancies in prediction accuracy compared to the previously published European data. Therefore, we performed the IrisPlex analysis on 65 Serbian individuals. Our results showed that the prediction of eye colour was accurate for about 57% of the respondents, which did not change significantly by applying the 0.7 threshold. The IrisPlex system performed well in predicting blue and brown eye colour in the Serbian sample, achieving high sensitivity levels. However, this model appeared to be unsuitable in the prediction of undefined eye colour, which was a common phenotype in our sample. This low sensitivity suggests a diverse and possibly population-specific

Corresponding author: Milica Keckarevic Markovic, Centre for Forensic and Applied Molecular Genetics, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia, milica@bio.bg.ac.rs, phone: +381653376868

genetic background of undefined eye colour. Given the large genetic diversity of the Serbian population, further work on a larger Serbian sample with more SNPs analysed is needed to reveal genetic variants mainly associated with this trait.

Keywords: DNA phenotyping, Eye colour prediction, IrisPlex, Forensic genetics, Serbian population

INTRODUCTION

In forensic casework, conventional STR profiling represents the gold standard of DNA analysis. However, its application is limited in situations where the DNA profile from the crime scene does not match the suspect's DNA profile or a profile from the DNA database. In this case, witness testimony about the appearance of perpetrator could be of a crucial importance for focusing the investigation on an appropriate group of people (WALSH *et al.*, 2011). Forensic DNA phenotyping is a very important field in the age of "DNA intelligence", because it offers the possibility of obtaining information about a person's externally visible characteristics (EVCs), such as eye, hair and skin colour, from DNA data alone (KAYSER and SCHNEIDER, 2009). Pigmentation traits are found to have an easily recognisable phenotype and strong genetic determinacy (STURM and FRUDAKIS, 2004). Information about EVCs comes from specific genetic markers called single nucleotide polymorphisms (SNPs), located within or near genes involved in determining certain trait. Overall, genotypes obtained in this way could not only confirm or negate witness statements about the phenotype of an unknown suspect but could even be the only evidence in the absence of such claims (DEMBINSKI and PICARD, 2014). Furthermore, this type of data could be useful in disasters or tragic events when identification of victims or missing persons is required without knowing their biogeographic origin (FRUDAKIS, 2008).

Human eye colour is one of the most studied polygenic traits (POŚPIECH *et al.*, 2011). It exhibits the highest degree of phenotypic variability in Europe, which is consistent with the Out-of-Africa hypothesis about the origin of modern humans, arguing that brown is the ancestral iris colour and that eye colour evolved on the European continent as a genetically and phenotypically variable trait from a small number of genetic founders with non-brown irises. (EIBERG *et al.*, 2008; WALSH *et al.*, 2012). Therefore, lighter eye colours are thought to be specific to European populations (EIBERG *et al.*, 2008; WALSH *et al.*, 2012). One of the assumptions is that the derived eye colours arose through a mechanism of positive selection due to greater mating preference. Alternatively, it has been proposed that variation in eye colour resulted from adaptation to environmental conditions with lower UV radiation and the consequent need for higher conversion of vitamin D (FROST, 2006; WALSH *et al.*, 2012). Consistent with this assumption, the presence of a north-south gradient in the geographic distribution of blue eye colour frequency in Europe, with the highest values in the southern Baltic region, could be a clear indication of the origin of this eye colour (FROST, 2006; WALSH *et al.*, 2012).

Many genome-wide association studies (GWAS), conducted primarily in European populations, have indicated the existence of a large number of variants associated with genes whose interactions contribute to eye colour variation in humans (EIBERG *et al.*, 2008; KAYSER *et al.*, 2008; HAN *et al.*, 2008; LIU *et al.*, 2009). According to current knowledge, the *HERC2* and *OCA2* genes have the greatest influence in determining iris colour (SULEM *et al.*, 2007; KAYSER *et al.*, 2008; WALSH *et al.*, 2012). The SNP rs12913832, located in a highly conserved intronic region of the *HERC2* gene, strongly affects melanin synthesis in human melanocytes by

regulating the expression of a nearby *OCA2* gene (STURM *et al.*, 2008). It has been shown that the rs12913832 T allele in *HERC2* leads to increased *OCA2* expression, resulting in brown eye colour in individuals carrying at least one such variant. On the other hand, the rs12913832 C allele leads to decreased *OCA2* expression, influencing blue eye colour in individuals carrying the homozygous genotype (STURM *et al.*, 2008; STURM *et al.*, 2009; VISSER *et al.*, 2012). In addition to these genes, there are several other genes found to have significantly smaller effect on eye colour determination, such as *SLC24A4* (HAN *et al.*, 2008; SULEM *et al.*, 2008), *SLC45A2* (HAN *et al.*, 2008), *TYR* (SULEM *et al.*, 2007), *TYRP1* (SULEM *et al.*, 2008), *IRF4* (HAN *et al.*, 2008), *ASIP* (KANETSKY *et al.*, 2002), *LYST* (LIU *et al.*, 2010) and *DSCR9* (LIU *et al.*, 2010). One of the studies conducted in 3804 Dutch Europeans showed that it is possible to achieve high predictive accuracy for blue and brown eye colour by typing only six informative SNPs from six genes well-associated with the trait (LIU *et al.*, 2009). Based on these findings, a 6-SNP multiplex SNapShot method, called the IrisPlex assay, was developed for the correct prediction of human eye colour from DNA samples (WALSH *et al.*, 2011).

The IrisPlex assay has been validated by several studies performed in several European populations (PURPS *et al.*, 2011; WALSH *et al.*, 2012; KASTELIC *et al.*, 2013; EDWARDS *et al*, 2016; SALVORO *et al.*, 2019; SALVO *et al.*, 2023), as well as in some Eurasian (YUN *et al.*, 2014; PRESTES *et al.*, 2011), Asian (EDWARDS *et al.*, 2016), US populations (DEMBINSKI and PICARD, 2014) and population of Argentina (HOHL *et al.*, 2022). However, a large amount of worldwide data is needed to confirm its applicability in forensic cases involving individuals with different geographic origins. Therefore, the aim of this work was to evaluate IrisPlex in the Serbian population and to determine if there are any possible discrepancies in prediction accuracy compared to previously published data.

MATERIALS AND METHODS

Study participants

Buccal swabs were obtained from 65 individuals for IrisPlex analysis. All procedures performed in this study were in accordance with the Helsinki declaration (1964) and its subsequent amendments and the study was approved by the authorities of the Faculty of Biology – University of Belgrade. Participants provided written informed consent and were asked about their gender, ancestry, and self-reported eye colour, which was divided into three generated categories (brown, undefined and blue). Undefined eye colour category included green eyes as well as eyes with different pigments that could not be clearly classified as brown or blue. Eye images were taken with a Panasonic DMC-FZ18 camera. Of the 65 analysed samples, 33 (51%) had brown, 23 (35%) had undefined and 9 individuals (14%) had blue eye phenotype.

Genetic analysis

DNA was extracted from the samples using the QIAamp® DNA Mini Kit (Qiagen, Hagen, Germany) according to the manufacturer's protocol. DNA quantification was performed with the Qubit™ dsDNA BR Assay Kit (Invitrogen, USA) following the manufacturer's instructions on a Qubit® 2.0 Fluorometer (Invitrogen, USA). Multiplex PCR was performed in a 15 μl volume containing 1.5μl 1× KAPA Taq Buffer A (containing 0.7μl 1.5mM MgCl2) (KapaBiosystems, USA), 0.45μl 200 μM mix of deoxyribonucleotides (dNTPs) (Fermantas,

Germany), 1.6µl primer mix, 0.06µl 0.02 U/µl Taq polymerase (KapaBiosystems, USA) and genomic DNA extract in different concentrations of 7–10 ng. Primer mix contained primers which were targeting six SNP markers of the six genes that regulate pigmentation: rs12913832 (*HERC2*), rs1800407 (*OCA2*), rs12896399 (*SLC4A4*), rs16891982 (*SLC45A2*), rs1393350 (*TYR*), and rs12203592 (*IRF4*). In this study, we used primers designed by WALSH *et al.* (2011). Sequences of primer pairs for *HERC2*, *TYR*, and *IRF4* and reverse primers for *SLC24A4* and *SLC45A2* were used without any adjustments, while the *OCA2* primer pair and forward primers for *SLC24A4* and *SLC45A2* were modified. Primer sequences and their final concentrations are listed in Table 1.

Table 1. Primer sequences

Gene	Forward and reverse PCR primer sequences	Primer concentration for multiplex PCR	Primer sequences with t-tail
HERC2	5' TGGCCTCTTGTGCTTGACTC 3'	1 µM	ttttttttttttttttttttt-
	5' GGCCCCTGATGATGATAGC 3'	1 µM	GCGTGCAGAACTTGACA
OCA2	5' TGACGTTGCTCAAGAA 3'	1 µM	tttttt-GGGAGAGCCGGTATGC
	5' TGTCTTACGAGCCTGCCTACT 3'	1 µM	
SLC24A4	5' GCATAGGGCATATTTAAGC 3'	1 µM	tttttttttttttttttttttttttttttt-
	5' CTTAGCCCTGGGCTTTGATG 3'	1 µM	CTTTTAGGCTAGTATATTTTGGG
SLC45A2	5' TGTGCTAGACCAGAAACTT 3'	1 µM	ttttttttt-AAACACGGAGTTGATGCA
	5' CGAAAGAGGAGCTGAGGTTG 3'	1 µM	
TYR	5' TCTCCTAGCTCTCTCTTGC 3'	0.7 µM	ttttttttttttttttttttt-
	5' GGGAAGGTGAATGATAACACG 3'	0.7 µM	TTTGTAAAAGACCACACAGATTT
IRF4	5' ACAGGGCAGCTGACTCTTCT 3'	0.4 µM	ttttttttttttttt-
	5' GCTAAACCTGGCACCAAAAG 3'	0.4 µM	TTTGGTGGGTAAAAGAAGG

PCR amplification was performed on the Veriti® Thermal Cycler (Applied Biosystems, USA) with the following thermocycling conditions: 97°C for 3 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min. After multiplex PCR, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. SNaPshot was performed using the ABI PRISM® SNaPshot™ Multiplex Kit (ThermoFisher Scientific, USA). SNaPshot reaction contained 1.5 µl of purified PCR product, 2.5 µl of SNaPshot reaction mixture, and 1 µl of primer mixture (with a primer concentration of 0.2 µM) in a total volume of 5 µl. Primer details can be found in Table 1. Thermocycling was performed on the Veriti® Thermal Cycler (Applied Biosystems, USA). The thermocycling programme was used under the same conditions as in WALSH *et al.* (2011): 96 °C for 2 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Capillary electrophoresis was performed using 2 µl of the purified SNaPshot products on the ABI-3130 Genetic Analyzer (Applied Biosystems, USA).

Data analysis

Data analysis was performed using GeneMapper software (Applied Biosystems, USA). Eye colour prediction was performed using IrisPlex software and HIrisPlex Eye and Hair Colour DNA Phenotyping Webtool (https://hirisplex.erasmusmc.nl/) based on multinomial logistic regression model previously published by LIU *et al.* (2009). Three prediction probability values (*p*) were generated as output data for each of the three defined phenotypes based on the predictive capability of the detected minor SNP alleles. The phenotype with the highest probability value was considered as the predicted iris colour. To avoid prediction errors, we additionally used the threshold of 0.7 recommended as most suitable by WALSH *et al.* (2012). In that case, only those *p* values above the defined threshold were considered appropriate for the eye colour prediction. Fisher's exact tests were performed to determine the statistical significance of differences between the present and previous studies, as well as the statistical significance of phenotype-genotype correlations.

RESULTS

Results of IrisPlex analysis of analysed samples are shown in Table 2.

Table 2. Detected genotypes of the six SNPs, eye colour phenotypes, predicted eye colours and prediction probabilities (p). Data also include prediction model performance with and without threshold of 0.7 applied.

a)

phenotype	Sample	rs12913832 (*HERC2*)	rs12203592 (*IRF4*)	rs1800407 (*OCA2*)	rs12896399 (*SLC24A4*)	rs16891982 (*SLC45A2*)	rs1393350 (*TYR*)	*p* for blue	*p* for undefined	*p* for brown	Prediction without threshold	Prediction performance without threshold	Prediction with threshold	Prediction performance with threshold
brown colour phenotype	IP#3	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#6	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#7	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#8	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#9	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#10	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#11	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#12	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#13	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#14	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#19	CC	CC	AA	GG	GG	CC	0.915	0.067	0.018	blue	x	blue	x
	IP#20	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#27	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#28	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#32	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	brown	✓	brown	✓
	IP#34	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	brown	✓	brown	✓
	IP#38	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#39	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	blue	✓	brown	✓
	IP#40	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#41	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#42	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	brown	✓	brown	✓
	IP#48	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#49	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#50	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#51	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#52	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	✓	brown	✓
	IP#53	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#56	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#73	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	✓	brown	✓
	IP#76	CT	CC	AA	GG	GG	CT	0.915	0.067	0.018	blue	x	blue	x
	IP#18	CC	CC	AA	GG	GG	CC	0.954	0.041	0.005	blue	x	blue	x
	IP#22	CT	CC	AA	GG	GG	CC	0.915	0.067	0.018	blue	x	blue	x
	IP#77	CT	CT	GA	GG	GG	CC	0.206	0.324	0.47	brown	✓	inconclusive	inconclusive

b)

phenotype	Sample	rs12913832 (*HERC2*)	rs12203592 (*IRF4*)	rs1800407 (*OCA2*)	rs12896399 (*SLC24A4*)	rs16891982 (*SLC45A2*)	rs1393350 (*TYR*)	*p* for blue	*p* for undefined	*p* for brown	Prediction without threshold	Prediction performance without threshold	Prediction with threshold	Prediction performance with threshold
undefined phenotype	IP#1	CC	CC	AA	GG	GG	CT	0.884	0.073	0.044	blue	x	blue	x
	IP#2	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#4	CT	CT	AA	GG	GG	CT	0.119	0.213	0.668	blue	x	inconclusive	inconclusive
	IP#5	CT	CC	AA	GG	GG	CC	0.143	0.227	0.629	brown	x	inconclusive	inconclusive
	IP#16	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	x	blue	x
	IP#21	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	brown	x	brown	x
	IP#23	CT	CC	AA	GG	GG	CC	0.143	0.227	0.629	brown	x	inconclusive	inconclusive
	IP#24	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	brown	x	brown	x
	IP#25	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#31	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#33	CT	CC	AA	GG	GG	CT	0.196	0.248	0.557	brown	x	inconclusive	inconclusive
	IP#35	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	x	blue	x
	IP#36	CT	CC	AA	GG	GG	CT	0.074	0.133	0.794	blue	x	brown	x
	IP#37	CT	CC	AA	GG	GG	CT	0.196	0.248	0.557	brown	x	inconclusive	inconclusive
	IP#44	CT	CC	AA	GG	GG	CC	0.143	0.227	0.629	brown	x	inconclusive	inconclusive
	IP#45	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	x	blue	x
	IP#46	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	x	blue	x
	IP#47	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	x	blue	x
	IP#55	CC	CC	AA	GG	GG	CT	0.884	0.073	0.044	blue	x	blue	x
	IP#61	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#62	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#63	CT	CC	AA	GG	GG	CC	0.05	0.114	0.836	brown	x	brown	x
	IP#68	CT	CT	AA	GG	GG	CT	0.119	0.213	0.668	brown	x	inconclusive	inconclusive

c)

phenotype	Sample	rs12913832 (*HERC2*)	rs12203592 (*IRF4*)	rs1800407 (*OCA2*)	rs12896399 (*SLC24A4*)	rs16891982 (*SLC45A2*)	rs1393350 (*TYR*)	*p* for blue	*p* for undefined	*p* for brown	Prediction without threshold	Prediction performance without threshold	Prediction with threshold	Prediction performance with threshold
blue colour phenotype	IP#15	TT	CC	AA	GG	GG	CC	0	0.013	0.986	brown	x	brown	x
	IP#29	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#30	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#43	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#64	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#69	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#70	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓
	IP#17	CC	CC	AA	GG	GG	CT	0.934	0.054	0.012	blue	✓	blue	✓
	IP#59	CC	CC	AA	GG	GG	CC	0.848	0.088	0.065	blue	✓	blue	✓

Analysis without threshold for brown eye phenotype showed correct call rate and model sensitivity of 88%, as 29 of 33 brown eye samples were predicted correctly. On the other hand, 17 false positive predictions were observed, corresponding to a specificity of 47%. After applying the threshold of 0.7, the results showed 28 true positive (85%) and 5 false negative predictions (15%). In this case, the false positive rate was lower as there were 10 false positive predictions. The achieved sensitivity and specificity of the model were 85% and 69%, respectively. In a group of participants with undefined eye colour, there were no correct predictions. When analysed without threshold, 7 samples were associated with blue colour (30%) and 16 samples with brown colour (70%). Using the threshold, 7 predictions were still made for blue eyes (30%), 9 predictions were made for brown eyes (40%) and 7 were inconclusive (30%).

Given these results, predictions for the undefined eye colour were present neither with nor without threshold. When analyzing the samples from participants with blue eyes, the sensitivity of the prediction model without threshold was 89%, as there were 8 true positive predictions. There were 11 false positive predictions reported, so the specificity was 80%. For these samples, the results of the analysis with the threshold showed the same sensitivity and specificity level as without the threshold.

Overall, the prediction model without threshold resulted in 57% correct and 43% incorrect predictions (Fig. 1a). Using the threshold, prediction was correct for 56%, incorrect for 32%, and inconclusive for 12% of the samples examined (Fig. 1b).

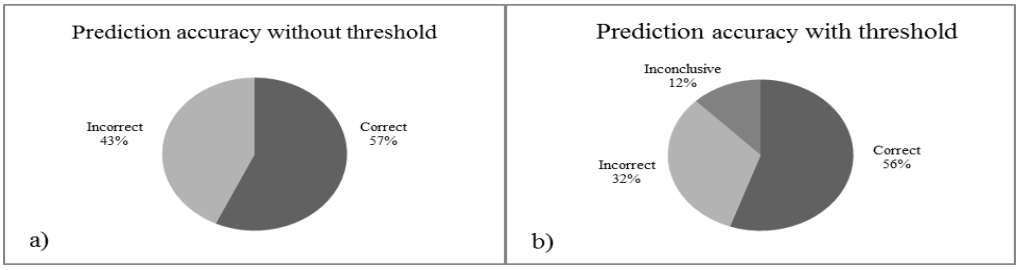

Figure 1. The IrisPlex prediction accuracy. (a) Accuracy of the prediction model without threshold, (b) accuracy of the prediction model with the 0.7 threshold.

The distribution of the genotypes of the four SNPs in three phenotype categories is shown in Table 3. SNP rs12913832 (*HERC2*) showed three genotypes (TT, CT and CC) in individuals with brown eye colour and two genotypes in individuals with undefined (CT and CC) and blue eye colour (CC and TT). Of the 33 individuals with brown eyes 21%, 73% and 6% carried TT, CT and CC, respectively. Among 23 individuals with undefined eyes, 30% were of CC and 70% of CT genotype. In 8 of 9 (89%) individuals with blue eyes CC homozygotes were observed, while one individual carried TT genotype. In rs1800407 (*OCA2*), only GG and GA genotypes were observed with the most abundant GG genotype present in 88%, 78% and 89% of individuals with brown, undefined, and blue eyes, respectively. In rs12203592 (*IF4*), the genotypes CC and CT were reported, with genotype CC present in all participants with blue eyes (100%), 97% of participants with brown eyes and 91% of participants from the undefined eye group. The TT genotype was not observed. In rs1393350 (*TYR*) the genotypes CC and CT were also observed. Most participants from all three colour categories were CC carriers: 88%, 61%, and 89% of individuals with brown, undefined, and blue eye phenotypes, respectively. Once again, the TT genotype was not observed. The markers rs12896399 (*SLC24A4*) and rs16891982 (*SLC45A2*) showed the same genotype (GG) in all participants.

Table 3. Distribution of SNP genotypes of the three genes regulating eye colour.

Eye colour	rs12913832 (*HERC2*)			rs1800407 (*OCA2*)			rs12203592 (*IRF4*)			rs1393350 (*TYR*)		
	TT	CT	CC	GG	GA	AA	TT	CT	CC	TT	CT	CC
Brown	21%	73%	6%	88%	12%	-	-	3%	97%	-	12%	88%
Undefined	-	70%	30%	78%	22%	-	-	9%	91%	-	39%	61%
Blue	11%	-	89%	89%	11%	-	-	-	100%	-	11%	89%

DISCUSSION

The human eye is an extremely complex structure whose colour is determined by the colour of the iris (LIU *et al.*, 2009). The greatest variation in eye colour has been found in European populations, which is in accordance with the hypothesis about the spread of modern humans and the evolutionary history of this trait (EIBERG *et al.*, 2008; WALSH *et al.*, 2012). It is therefore not surprising that many DNA-based eye colour prediction models were initially developed using European populations (LIU *et al.*, 2009). However, it is important to examine whether the varieties and sample size of the selected populations are sufficiently representative of Europe as a whole, considering its high degree of genetic polymorphism. In the present study, an analysis of the IrisPlex system containing six most informative SNPs for predicting human eye colour in Europeans (WALSH *et al.*, 2011) was performed on 65 Serbian respondents to evaluate its effectiveness and potential applicability in forensic investigations in Serbia.

Considering all three phenotypes - brown, undefined and blue eye colour - the application of six markers in the IrisPlex assay in our sample resulted in a moderately good prediction accuracy of 57% (Fig. 1a). This result is statistically significantly lower ($p \leq 0.0451$) than that previously reported for seven European populations from the EUREYE study (WALSH *et al.*, 2012). On the other hand, the IrisPlex system performed well in predicting brown and blue eye colour in our sample, resulting in sensitivities of 88% and 89%, respectively. However, this prediction model was not able to achieve accurate predictions for undefined eye colour, as was the case in several other studied populations (PRESTES *et al.*, 2014; DEMBINSKI and PICARD, 2014; KASTELIC *et al.*, 2013, HOHL *et al.*, 2022). If the undefined category was excluded from our study, the error rate in eye colour prediction would decrease from 43% to 12%, which is only slightly lower than the value reported in the EUREYE study (WALSH *et al.*, 2012). The higher overall prediction error rate in our case could therefore be explained by the fact that, compared to the 7% of the sample of WALSH *et al.* (2012), a significant proportion of our sample (35%) was phenotypically characterised by an undefined eye colour, which proved to be unpredictable according to our results (Table 2b). The complete lack of sensitivity in predicting undefined eye colours may reflect a high degree of admixture in the population, as previously mentioned in studies of Italian (SALVORO *et al.*, 2019), European-Asian (PRESTES *et al.*, 2014), and U.S. admixed populations (DEMBINSKI and PICARD, 2014).

When the threshold $p \geq 0.7$ was applied, the prediction specificity for brown eye colour increased as the false positive rate decreased, while the prediction accuracy slightly decreased. In contrast, the prediction specificity and sensitivity for blue eye colour did not change. However, in this case the overall frequency of incorrect predictions was lower, as all phenotypes with probability values below 0.7 were classified as "inconclusive" (Fig. 1b). This category mainly included individuals with green eye colour and individuals with the central heterochromia, characterised by a lighter iris with a darker ring around the pupil (MACKEY *et al.*, 2011). All respondents with "inconclusive" and approximately half of those with incorrectly predicted phenotype were heterozygous for rs12913832 in the *HERC2* gene (Table 2).

Our results confirm previous findings that much of the variability in eye colour can be explained by the SNP rs12913832 of the *HERC2* gene (STURM *et al.*, 2008). The largest number of participants with genotypes TT and CT for this SNP in our study had brown or undefined eye colour, whereas participants with genotype CC predominantly had blue eyes (Table 2). These

results are largely consistent with the assumption that the ancestral T allele on the *HERC2* gene is dominant and that the presence of at least one such variant is sufficient for the expression of a darker eye colour (EDWARDS *et al.*, 2016). However, a deviation from this regularity of prediction was observed in one individual with genotype TT, who clearly had blue eyes (Table 2c), and in two individuals with genotype CC, who clearly had brown eyes (Table 2a). This is not the first time that such a deficiency of the prediction model has been described (PRESTES *et al.*, 2014; DEMBINSKI and PICARD, 2014). The discrepancy found in a blue-eyed individual with the TT genotype was also documented in a study of Norwegians, according to which this phenomenon could be explained by the newly discovered blue-eyed variants in the *OCA2-HERC2* region (SALVO *et al.*, 2023). In addition, the occurrence of brown eye colour in the absence of the T allele in the SNP rs12913832 suggests that the darker eye colour may also be determined by other genes whose effects are likely to be additive. Apart from the *HERC2* gene, small variations in the genotype in our sample were detected also in the *OCA2*, *TYR* and *IRF4* genes (Table 3), which are thought to have an additive effect on eye colour variability. In our study, a 44% of undefined-eyed individuals who carried the CT genotype for *HERC2* also carried CT genotype for *TYR* gene, assumed to be associated to lighter eye colour (BOROVANSKY and RILEY, 2011), while only 17% of brown-eyed individuals who carried the CT genotype for *HERC2* also carried the CT genotype for *TYR* gene (Table 2). The correlation between the heterozygous genotype of both of *HERC2* and *TYR* genes and the lighter eye colour phenotype was examined in the brown and intermediate categories in order to confirm appearance of this type of genotype-phenotype association. The difference between two categories above was not statistically significant (p=0.08), probably due to the small number of participants in both groups, but it could be assumed that the shade of the undefined eye colour in some cases could be attributed to the combination of the genotypes of these two genes. The *OCA2* and *IRF* genes were predominantly represented with the major genotypes regardless of eye colour (Table 3), whereas the *SLC24A4* and *SLC45A2* genes had the same genotype in all participants (Table 2), suggesting that these four genes had an overall weak influence on the trait in our sample.

The difficulties in predicting undefined eye colours strongly suggest that the markers included in IrisPlex are not sufficiently effective in predicting the different shades between blue and brown colour. This could be caused by the fact that this prediction model does not take into account the possibility of gene epistasis, which is thought to have a major impact on the expression of undefined eye colour (POŚPIECH *et al.*, 2011; SALVORO *et al*, 2019). Furthermore, discrepancies in the interpretation of this colour between different studies (WALSH *et al.*, 2012; SALVORO *et a.l* 2019; KASTELIC *et al*., 2013; YUN *et al.*, 2014; PRESTES *et al.*, 2011; DEMBINSKI and PICARD, 2014; EDWARDS *et al.*, 2016; HOHL *et al.*, 2022), even in the same population (WALSH *et al.*, 2012; SALVORO *et al.*, 2019), were found, which could also be due to the subjective perception of observers and the lack of clear criteria for the determination of undefined eye colour. Additionally, the genetic background of various structures observed on the eye (Fuch's crypts, dark pigment spots, and contraction furrows) that influence the perception of eye colour is largely unknown. The lower predictive power of the IrisPlex system could therefore be caused by the presence of additional genetic determinants, the effect of which could be population-specific. The identification of genetic markers associated with different structures of the eye, as well as other pigmentation markers, could further improve eye colour prediction

models. On the other hand, the problem of objective classification of eye colour may soon be overcomed by the introduction of new digital models based on the use of high-resolution devices that can divide the eye image into many small parts counted as pixels (LIU *et al.*, 2010; ANDERSEN *et al.*, 2013; EDWARDS *et al.*, 2016; WOLLSTEIN *et al.*, 2017, PAPARAZZO *et al.*, 2022). However, in order to be applicable in forensic investigations, this categorisation of eye colour should be consistent with the subjective perception of the eyewitnesses.

CONCLUSIONS

Our study provides an overview of the distribution of genotypes for six SNP markers in the Serbian population which were found to be the most informative in predicting eye colour in European populations. The IrisPlex system performed well in predicting blue and brown eye colour in the Serbian sample, achieving a sensitivity of over 85%. However, it showed limitations in predicting undefined eye colour, the common phenotype in our sample. This lack of predictive sensitivity of the IrisPlex system to the undefined phenotype was particularly striking in the Serbian population, which is thought to have greater genetic diversity than most European populations due to its geographic location, turbulent history, and high degree of admixture. Thus, our results suggest that undefined eye colour is strongly dependent on unknown genetic factors in addition to the *HERC2* and probably also the *TYR* gene, the effects of which may be population-specific. However, further work with a larger sample, more genetic variants, and a model that accounts for epistatic interactions is needed to achieve sufficiently good prediction of undefined eye colour.

ACKNOWLEDGMENTS

The research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement number: 451-03-47/2023-01/ 200178).

Received, May 05th, 2023

Accepted September 01th, 2023

REFERENCES

ANDERSEN, J. D., P. JOHANSEN, S. HARDER, S. R. CHRISTOFFERSEN, M. C. DELGADO, S. T.HENRIKSEN, M. M. NIELSEN, E. SORENSEN, H. ULLUM, T. HANSEN, A. L.DAHL, R. R. PAULSEN, C. BORSTING, N. MORLING (2013): Genetic analyses of the human eye colours using a novel objective method for eye colour classification. Forensic Sci. Int. Genet., *7*(5), 508-515.

BOROVANSKY, J., P.A .RILEY (Eds.). (2011): Melanins and melanosomes:biosynthesis,structure, physiological and pathological functions. John Wiley & Sons, Hoboken, NJ.

DEMBINSKI, G. M., C. J. PICARD, (2014): Evaluation of the IrisPlex DNA-based eye colour prediction assay in a United States population. Forensic Sci. Int. Genet., *9*, 111-117.

EDWARDS, M., D. CHA, S. KRITHIKA, M., JOHNSON, G. COOK, E. J. PARRA, (2016): Iris pigmentation as a quantitative trait: variation in populations of European, East Asian and South Asian ancestry and association with candidate gene polymorphisms. Pigment Cell Melanoma Res., *29*(2), 141-162.

EIBERG, H., J. TROELSEN, M. NIELSEN, A. MIKKELSEN, J. MENGEL-FROM, K. W. KJAER, L. HANSEN (2008): Blue eye colour in humans may be caused by a perfectly associated founder mutation in a regulatory element located within the HERC2 gene inhibiting OCA2 expression. Hum. Genet., *123*(2), 177-187.

FROST, P. (2006): European hair and eye colour: a case of frequency-dependent sexual selection?. Evol.Hum.Behav., *27*(2), 85-103.

FRUDAKIS, T. N. (Ed.). (2008): Molecular Photofitting: Predicting Ancestry and Phenotype from DNA. (1st ed.) Academic Press, Burlington, MA.

HAN, J., P. KRAFT, H. NAN, Q. GUO, C. CHEN, A. QURESHI, S. E. HANKINSON, F. B. HU, D. L. DUFFY, Z. Z. ZHAO, N. G. MARTIN, G. W. MONTGOMERY, N. K.HAYWARD, G. THOMAS, R. N. HOOVER, S. CHANOCK, D. J. HUNTER(2008): A genome-wide association study identifies novel alleles associated with hair colour and skin pigmentation. PLoS Genet., *4*(5), e1000074.

HOHL, D. M., R. GONZÁLEZ, G. P. DI SANTO MEZTLER, J. PATINO-RICO, C. DEJEAN, S.AVENA, M. DE LOS ÁNGELES GUTIÉRREZ, C. I. CATANESI (2022): Applicability of the IrisPlex system for eye colour prediction in an admixed population from Argentina. Ann. Hum. Genet., *86*(6), 297-327.

KANETSKY, P. A., J. SWOYER, S.,PANOSSIAN, R. HOLMES, D, GUERRY, T. R. REBBECK (2002):A polymorphism in the agouti signaling protein gene is associated with human pigmentation. Am. J. Hum. Genet., *70*(3), 770-775.

KASTELIC, V., E. POŚPIECH, J. DRAUS-BARINI, W. BRANICKI, K. DROBNIČ (2013): Prediction of eye colour in the Slovenian population using the IrisPlex SNPs. Croat. Med. J., *54*(4), 381-386.

KAYSER, M., F. LIU, A. C. J. JANSSENS, F. RIVADENEIRA, O. LAO, K., VAN DUIJN, M.VERMEULEN, P. ARP, M. M. JHAMAI, W. F. J. VAN IJCKEN DEN DUNNEN, J. T. S. HEATH, D. ZELENIKA,. D. G. DESPRIET, DC. C. W. KLAVER,J. R. VINGERLING, P. T. V. M. DE JONG, A. HOFMAN, Y. S. AULCHENKO, A. G.UITTERLINDEN, B. A. OOSTRA, C. M. VAN DUIJN (2008): Three genome-wide association studies and a linkage analysis identify HERC2 as a human iris colourgene. Am. J. Hum. Genet., *82*(2), 411-423.

KAYSER, M., P. M. SCHNEIDER (2009): DNA-based prediction of human externally visible characteristics in forensics: motivations, scientific challenges, and ethical considerations. Forensic Sci. Int. Genet., *3*(3), 154-161.

LIU, F., K. VAN DUIJN, J. R. VINGERLING, A. HOFMAN, A. G. UITTERLINDEN, A. C. J. JANSSENS, M.KAYSER (2009): Eye colour and the prediction of complex phenotypes from genotypes. Curr. Biol., *19*(5), R192-R193.

LIU, F., 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 (2010): Digital quantification of human eye colour highlights genetic association of three new loci. PLoS Genet., *6*(5), e1000934.

MACKEY, D. A., C. H. WILKINSON, L. S.,KEARNS, A. W. HEWITT (2011): Classification of iris colour: review and refinement of a classification schema. Clin. Experiment. Ophthalmol., 39(5), 462-471.

PAPARAZZO, E., A. GOZALISHVILI, V. LAGANI, S. GERACITANO, A. BAULEO,E.FALCONE,G. PASSARINO, A.MONTESANTO (2022): A new approach to broaden the range of eye colour identifiable by IrisPlex in DNA phenotyping. Sci. Rep., *12*(1), 12803.

POŚPIECH, E., J. DRAUS-BARINI, T. KUPIEC, A. WOJAS-PELC, W.BRANICKI (2011): Gene–gene interactions contribute to eye colour variation in humans. J. Hum.Genet., *56*(6), 447-455.

PRESTES, P. R., R. J. MICTHELL, R. DANIEL, K. N. BALLANTYNE, R. A. H. VAN OORSCHOT (2011): Evaluation of the IrisPlex system in admixed individuals. Forensic Sci. Int. Genet. Suppl. Ser., *3*(1), e283-e284.

PURPS, J., M. GEPPERT, M. NAGY, L. ROEWER (2011): Evaluation of the IrisPlex eye colour prediction tool in a German population sample. Forensic Sci. Int. Genet. Suppl. Ser., *3*(1), e202-e203.

SALVO, N. M., J. D. ANDERSEN, K. JANSSEN, O. L. MEYER, T. BERG, C. BØRSTING, G-H.OLSEN (2023): Association between Variants in the *OCA2-HERC2* Region and Blue Eye Colour in *HERC2* rs12913832 AA and AG Individuals. Genes, *14*(3), 698.

SALVORO, C., C. FACCINETTO, L. ZUCCHELLI, PORTO, M., MARINO, A., OCCHI, G.,GUSTAVO DE LOS CAMPOS, G.VAZZA (2019): Performance of four models for eye colour prediction in an Italian population sample. Forensic Sci. Int. Genet., *40*, 192-200.

STURM, R. A., T. N. FRUDAKIS (2004): Eye colour: portals into pigmentation genes and ancestry. Trends Genet., *20*(8), 327-332.

STURM, R. A., D. L. DUFFY, Z. Z. ZHAO, F. P. LEITE, M. S. STARK, N. K. HAYWARD, N. G.MARTIN, G. W. MONTGOMERY (2008): A single SNP in an evolutionary conserved region within intron 86 of the HERC2 gene determines human blue-brown eye colour. Am. J. Hum. Genet., *82*(2), 424-431.

STURM, R. A., M. LARSSON (2009): Genetics of human iris colour and patterns. Pigment Cell Melanoma Res., *22*(5), 544-562.

SULEM, P., D. F. GUDBJARTSSON, S. N. STACEY, A, HELGASON, T. RAFNAR,K. P. MAGNUSSON, A. MANOLESCU, A. KARASON, A. PALSSON,G. THORLEIFSSON, M. JAKOBSDOTTIR, S. STEINBERG, S, PÁLSSON,F. JONASSON, B. SIGURGEIRSSON, K. THORISDOTTIR, R. RAGNARSSON,K. R. BENEDIKTSDOTTIR, K. K. ABEN, L. A. KIEMENEY, J. H. OLAFSSON,J.GULCHER,A. KONG, U. THORSTEINDOTTIR, K.STEFANSSON (2007): Genetic determinants of hair, eye and skin pigmentation in Europeans. Nat. Genet., *39*(12), 1443-1452.

SULEM, P., D. F. GUDBJARTSSON, S. N. STACEY, A. HELGASON, T. RAFNAR,M. JAKOBSDOTTIR, S. STEINBERG, S. A. GUDJONSSON, A. PALSSON,G. THORLEIFSSON, S. PÁLSSON, B. SIGURGEIRSSON, K. THORISDOTTIR,R. RAGNARSSON, K. R. BENEDIKTSDOTTIR, K. K. ABEN, S. H. VERMEULEN,A. M. GOLDSTEIN, M. A. TUCKER, L. A. KIEMENEY, J. H. OLAFSSON,J. GULCHER, A. KONG, U. THORSTEINDOTTIR, K.STEFANSSON (2008): Two newly identified genetic determinants of pigmentation in Europeans. Nat. Genet., *40*(7), 835-837.

VISSER, M., M., KAYSER, R. J. PALSTRA (2012): HERC2 rs12913832 modulates human pigmentation by attenuating chromatin-loop formation between a long-range enhancer and the OCA2 promoter. Genome Res., *22*(3), 446-455.

WALSH, S., F. LIU, K. N. BALLANTYNE, M. VAN OVEN, O. LAO, M.KAYSER (2011): IrisPlex: a sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Sci. Int. Genet., *5*(3), 170-180.

WALSH, S., A, WOLLSTEIN, F. LIU, U. CHAKRAVARTHY, M. RAHU, J. H. SELAND, G., L. SOUBRANE, TOMAZZOLI, F. TOPOUZIS, J. R. VINGERLING, J. VIOQUE,A. E. FLECTHER, K. N. BALLANTYNE, M. KAYSER (2012): DNA-based eye colour prediction across Europe with the IrisPlex system. Forensic Sci. Int. Genet., *6*(3), 330-340.

WOLLSTEIN, A., S. WALSH, F. LIU, U. CHAKRAVARTHY, M. RAHU, J. H. SELAND,G. SOUBRANE, L. TOMAZZOLI, F. TOPOUZIS, J. R. VINGERLING, J. VIOQUE,S. BÖHRINGER, A. E. FLECTHER, M. KAYSER (2017): Novel quantitative pigmentation phenotyping enhances genetic association, epistasis, and prediction of human eye colour. Sci. Rep., *7*(1), 43359.

YUN, L., Y. GU, H. RAJEEVAN, K. K. KIDD (2014): Application of six IrisPlex SNPs and comparison of two eye colour prediction systems in diverse Eurasia populations. Int. J. Legal Med., *128*, 447-453.

EVALUACIJA IRISPLEX SISTEMA ZA PREDIKCIJU BOJE OČIJU U SRPSKOJ POPULACIJI

Marija VUKOVIĆ*, Milica MIHAJLOVIĆ SREJIĆ*, Katarina ŠAPIĆ, Vanja TANASIĆ, Verica RADOJIČIĆ, Milica KECKAREVIĆ MARKOVIĆ, Miljana KECMANOVIĆ, Dušan KECKAREVIĆ

Centar za forenzičku i primenjenu molekularnu genetiku, Biološki fakultet, Univerzitet u Beogradu, Beograd, Srbija
*podjednako doprineli

Izvod

Predikcija fizičkog izgleda na osnovu analize DNK, takođe poznata kao DNK fenotipizacija, je polje koje se brzo razvija i poseduje značajan potencijal u rešavanju zahtevnih forenzičkih istraga. Spoljašnje karakteristike, kao što je boja očiju, su lako uočljive i genetički determinisane. Analiza visoko informativnih pojedinačnih nukleotidnih polimorfizama (*eng. single nucleotide polymorphisms - SNP*) lociranih u regionima gena asociranih sa pomenutim osobinama, pruža informacije o fenotipu nepoznate individue, što može biti ključno u slučajevima kada analiza pomoću standardnih STR lokusa nije informativna. *IrisPlex* esej je razvijen na uzorku 3804 ispitanika populacije Holandije kako bi se precizno predvidela braon, plava i prelazna boja očiju na osnovu šest *SNP* markera. Esej je validiran od strane nekoliko studija i pokazana je velika preciznost u predikciji braon i plave boje očiju u većini evropskih populacija. Cilj ove studije bila je evaluacija *IrisPlex* eseja u srpskoj populaciji i utvrđivanje potencijalnih odstupanja u preciznosti predikcije u poređenju sa prethodno objavljenim evropskim podacima. Sprovedena je analiza 65 ispitanika srpske populacije. Dobijeni rezultati su pokazali da je predikcija bila tačna za nešto više od polovine ispitanika. *IrisPlex* esej je imao veoma dobre performanse u prediktovanju plave i braon boje očiju. Sa druge strane, model se pokazao kao neefikasan u predikciji prelazne boje što sugeriše da je genetička osnova ovog fenotipa u Evropi nepoznata i moguće populaciono-specifična. Uzimajući u obzir veliki genetički diverzitet srpske populacije, potreban je dalji rad na većem uzorku i sa više SNP markera kako bi se identifikovale sve genetičke varijante koje su u osnovi ove osobine.

Primljeno 05.V.2023.
Odobreno 1. IX 2023.