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Experimental long-distance haplotyping of *OCA2-HERC2* variants

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ABSTRACT

The regulatory *HERC2* SNP, rs12913832, is strongly associated with blue and brown eye colour. However, eye colour in heterozygous rs12913832 individuals is observed to vary greatly. Missense mutations in *OCA2,* such as rs1800407 and rs74653330, are associated with lighter eye colour in some but not all heterozygous rs12913832 individuals. Determining the physical linkage of these variants might help to further explain eye colour variation. So far, experimental haplotyping of these variants has been challenging because the genomic distance between them (~ 135 kb) exceeds the fragment lengths produced by commonly used DNA isolation kits. The aim for this study was to explore novel methods for long distance haplotyping to assess associations between *OCA2-HERC2* haplotypes and eye colour. DNA was isolated from frozen blood samples collected from Norwegians that are known to be heterozygous for both *HERC2* rs12913832 and *OCA2* SNPs, either rs1800407 (n = 23) or rs74653330 (n = 17), using the newly commercially available Monarch® HMW (heigh molecular weight) DNA Extraction Kit (New England BioLabs$_{inc}$). We successfully isolated DNA fragments up to 210 kb, which were long enough to haplotype *OCA2-HERC2* loci by droplet digital PCR (ddPCR). Three haplotypes were observed in the study population: rs12913832:A-rs1800407:T in 22/23 individuals, rs12913832:A-rs1800407:C in 1/23 individuals and rs12913832:A-rs74653330:T in 16/16 individuals. As expected, all individuals with the rs12913832:A-rs74653330:T haplotype had intermediate to blue eye colour. However, the rs12913832:A-rs1800407:T haplotype was observed in both blue and brown-eyed individuals, suggesting more research is needed.

1. Introduction

The *HERC2* SNP rs12913832 (enhancer region of *OCA2*) is the main predictor of blue and brown eye colour [1], especially in the homozygous state (AA and GG). However, eye colour in heterozygous rs12913832 individuals is observed to vary greatly [2]. Missense mutations in *OCA2* (e.g. rs1800407:T and rs74653330:T) are associated with lighter eye colour in some but not all rs12913832:AG Scandinavians [2–4]. Thus, the physical linkage of *HERC2* rs12913832 and *OCA2* variants may further explain eye colour variation. So far, experimental long distance haplotyping has been technically challenging, because the genomic distance between these loci (~135 kb) is much greater than the DNA fragments size produced by commonly used DNA isolation kits. In this study, we aim to demonstrate the feasibility of isolating long DNA fragments for long distance haplotyping by ddPCR and use this methodology to assess associations between *OCA2-HERC2* haplotypes and eye colour.

2. Material and methods

Frozen blood samples from 40 rs12913832:AG Norwegians with either rs1800407:CT (n = 23) or rs74653330:CT (n = 17) were selected for experimental haplotyping from a database of 545 Norwegians, described in previous studies [2,3]. Eye colour varied from blue to brown (PIE-score [5]: from −1 to 1). The project is approved by the Faculty of Health Sciences, UiT - The Arctic University of Norway (reference number 2021/2034).

DNA was isolated using the newly commercially available Monarch® HMW DNA Extraction Kit (New England BioLabs$_{inc}$). DNA fragment length was tested with ddPCR using the “mile marker” assays, targeting the *RPP30* locus, as described in Regan et al., 2015 [6]. Assays are FAM-labelled and target sequences at distances from 1 to 210 kb from an HEX-labelled anchor assay. As a negative control the “mile marker” assays were also haplotyped with another anchor, on the *EIF2C1* locus, residing on a different chromosome.

Individuals were haplotyped by ddPCR using Bio-Rad’s QX200

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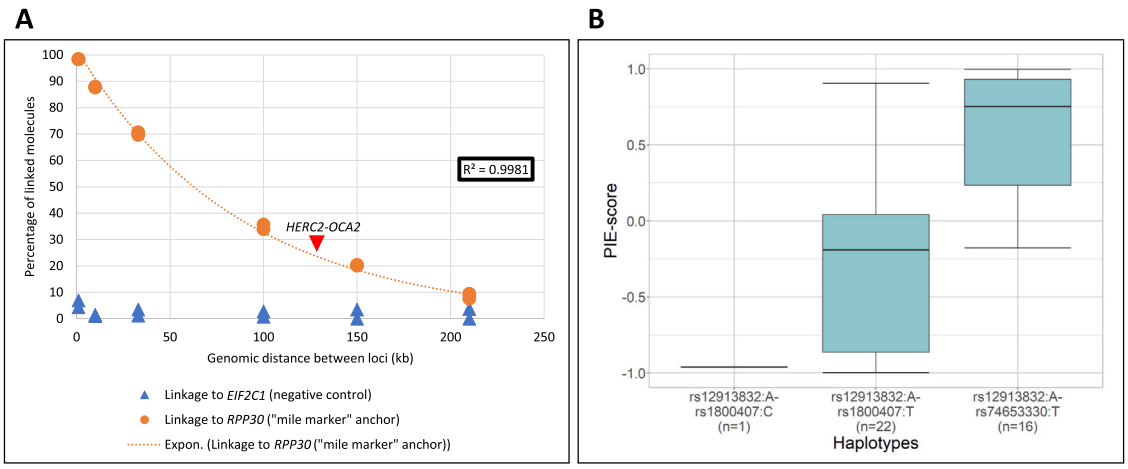

Fig. 1. A) DNA fragment lengths in Monarch® extracted HMW DNA visualised by plotting percentage of linked loci after ddPCR against genomic distance of "mile marker" assays. ▼ genomic distance between OCA2-HERC2 targets. **B)** Haplotypes and Pixel Index of the Eye (PIE)-scores from − 1 (brown) to 1 (blue), in a Norwegian study population (n = 39).

ddPCR system and the phasing protocol by Regan and Karlin-Neumann, 2018 [7]. Only two targets can be haplotyped in one analysis. Thus, to cover all possible allele combinations, four unique HEX-FAM duplexes per marker pair were designed by Bio-Rad (assay ID dHsaMDM2285425001/dHsaMDW2285425003 for rs12913832, assay ID dHsaMDW8577445873/dHsaMDM8577445871 for rs1800407 and assay ID dMDM1502681141/dMDW1502681143 for rs74653330). Primers and Iowa Black quenched probes were designed with an optimal annealing temperature of 55°C, and standard cycling conditions for ddPCR. To minimise cross-reaction, dark competitor probes for each assay were added. Data was analysed for linkage of markers with QX Manager v.1.2.

3. Results and discussion

We successfully isolated DNA fragments up to 210 kb, which was long enough for experimental haplotyping of *OCA2-HERC2* (Fig. 1A). Additionally, by using the "mile marker" assays, we observe that over 20% of the fragments were longer than 135 kb (▼ in Fig. 1A), clearly distinguishable from the background (▲ in Fig. 1A). Thus, variants of interest could successfully be phased by ddPCR. Notably, care should be taken when working with HMW DNA due to susceptibility to fragmentation and sample viscosity. Long DNA fragments are more difficult to mix to homogeneity due to entangling and might therefore compromise droplet generation. Hence, we used 20 ng input DNA per reaction instead of the recommended 40 ng [7]. When experimentally haplotyping *OCA2-HERC2* in the study population, linkage down to 5% was observed. This was possibly due to low DNA concentration and fragmentation in the respective samples as the blood had been stored for up to seven years. However, by utilising all four duplexes, all but one sample could be haplotyped with confidence.

Successful haplotyping in 39 Norwegians revealed three haplotypes (Fig. 1B): rs12913832:A-rs1800407:T in 22/23 individuals, rs12913832:A-rs1800407:C in 1/23 individuals and rs12913832:A-rs74653330:T in 16/16 individuals. This means that all but one individual carried the derived G-allele in rs12913832 in *trans*-phase with either the *OCA2* missense mutations rs1800407:T or rs74653330:T. *OCA2* expression is reduced in the presence of rs12913832:G [8]. Hence, it was expected to find these haplotypes in intermediate and blue-eyed individuals as they have one protein at reduced production and one variant protein. This agrees with the observation that all rs12913832:A-rs74653330:T individuals had intermediate and blue eyes. Surprisingly, eye colour in rs12913832:A-rs1800407:T individuals varied from blue to brown, questioning the effect of rs1800407 on eye colour in the Norwegian population. Only one brown eyed individual had the expected rs12913832:A-rs1800407:C haplotype. rs1800407 is included in the forensically validated prediction tool, IrisPlex, and has been reported to have low, but measurable effect on eye colour in several populations [5,9]. Association of the rs1800407 and eye colour has also been observed in the Norwegian population, but not on a rs12913832:AG and AA background [2]. We suggest to haplotype these SNPs in other populations as well to get a deeper understanding of their impact on eye colour.

4. Conclusion

Phasing by ddPCR was rapid and enabled screening of particular *OCA2-HERC2* haplotypes in a Norwegian study population. The newly available Monarch® HMW DNA Extraction Kit was an excellent choice for isolating long DNA fragments, even from frozen blood samples, making long-distance haplotyping possible. As expected, all rs74653330:T individuals had intermediate and blue eyes and had the derived T-allele in trans-phase with the derived G-allele in rs12913832. However, the rs12913832:A-rs1800407:T haplotype was observed in individuals with varying eye colour (from blue to brown), suggesting more research is needed to better understand normal eye colour variation.

Declaration of Competing Interest

None.

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