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RESEARCH ARTICLE

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Genome-wide SNP typing of ancient DNA: Determination of hair and eye color of Bronze Age humans from their skeletal remains

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Abstract

Objective: A genome-wide high-throughput single nucleotide polymorphism (SNP) typing method was tested with respect of the applicability to ancient and degraded DNA. The results were compared to mini-sequencing data achieved through single base extension (SBE) typing. The SNPs chosen for the study allow to determine the hair colors and eye colors of humans.

Material and methods: The DNA samples were extracted from the skeletal remains of 59 human individuals dating back to the Late Bronze Age. The 3,000 years old bones had been discovered in the Lichtenstein Cave in Lower Saxony, Germany. The simultaneous typing of 24 SNPs for each of the ancient DNA samples was carried out using the 192.24 Dynamic Array™ by Fluidigm®.

Results: Thirty-eight of the ancient samples (=64%) revealed full and reproducible SNP genotypes allowing hair and eye color phenotyping. In 10 samples (=17%) at least half of the SNPs were unambiguously determined, in 11 samples (=19%) the SNP typing failed. For 23 of the 59 individuals, a comparison of the SNP typing results with genotypes from an earlier performed SBE typing approach was possible. The comparison confirmed the full concordance of the results for 90% of the SNP typings. In the remaining 10% allelic dropouts were identified.

Discussion: The high genotyping success rate could be achieved by introducing modifications to the preamplification protocol mainly by increasing the DNA input and the amplification cycle number. The occurrence of allelic dropouts indicates that a further increase of DNA input to the preamplification step is desirable.

KEYWORDS

ancient and degraded DNA, eye color, genome wide, hair color, high-throughput SNP typing, phenotype

1 | INTRODUCTION

Knowledge of our ancestor's outer appearance helps to understand where we come from and the complex process of human migration, pigmentation, and adaptive processes to different climatic conditions (Parra, 2007). In forensic investigations, another application of genotyping in the form of phenotyping can be found. Forensic DNA phenotyping (FDP) enables to identify external visible characteristics even of trace material for example, of crime scenes (Kayser, 2015; Kayser & Schneider, 2009; Maroñas et al., 2015).

Single nucleotide polymorphisms (SNPs) are the favored genetic markers used for forensic DNA phenotyping approaches (Fareed & Afzal, 2013). During the last years it was possible to identify DNA markers that are highly predictive for eye and hair color (Draus-Barini et al., 2013; Kayser, 2013; Maroñas et al., 2015). Besides a variety of different phenotyping kits which can be used for eye and hair color prediction (Draus-Barini et al., 2013; Walsh et al., 2013) there is also a multitude of methods for analyzing SNPs. When it comes to analyzing degraded DNA samples, the fragmentation of the DNA is a major obstacle. During the degradation process, the double helix of the DNA gets increasingly fragmented, thereby the amount of intact targets is being reduced which may lead to amplification difficulties or even amplification failure (Alaeddini, Walsh, & Abbas, 2010; Fondevila et al., 2008).

Particular in forensic contexts high levels of DNA degradation constitute an obstacle for multiplex autosomal STR-typing which had first been used to genetically fingerprint degraded skeletal material in the early 1990s (e.g., Jeffreys, Allen, Hagelberg, & Sonnberg, 1992). Characteristic for samples revealing incomplete STR profiles is the full presence of smaller fragments up to 200 bp while fragments exceeding 300 bp fail to amplify (Butler, 2001; Hummel, 2003). This problem was overcome by the introduction of so called mini-STR kits with target sizes of up to 280 bp (Hellmann, Rohleder, Schmitter, & Wittig, 2001; Hughes-Stamm, Ashton, & van Daal, 2011; Seidenberg et al., 2012).

Due to comparatively short target sizes the different SNP typing methods, however, are not affected by DNA degradation to the same extend as it applies for multiplex STR typing. In a unique manner, this holds true for the comparatively new next generation sequencing (NGS) technique which allows the acquisition of whole genome data even from strongly fragmented DNA in high throughput approaches (for a review cf. Hofreiter et al., 2015). However, if a restriction to certain genetic information is sufficient and a lesser bioinformatic expenditure as necessary for the handling of NGS data is aimed still RFLP analyses and standard Sanger sequencing, particularly the single base extension (SBE) technique which is also called SNaPshot™ (Applied Biosystems) or Mini-Sequencing are suitable for ancient and degraded DNA analysis. Particularly, the SBE technique proved to be extremely sensitive, robust, and reliable (Sobrino, Brión, & Carracedo, 2005). By using the different SNP-genotyping techniques, it was for example possible to successfully investigate Y-chromosomal SNPs from degraded and ancient DNA material (Bouakaze, Keyser, Amory, Crubézy, & Ludes, 2007; Lessig et al., 2005; Petkovski, Keyser-Tracqui, Crubézy, Hienne, & Ludes, 2006). SNP arrays are particularly known for being a high-throughput method which requires only small amounts of DNA. Although they are so far commonly used for the analyzing of modern DNA material, they have not been often applied to ancient DNA samples (Cho, Seo, Lee, Yu, & Lee, 2016; Mead et al., 2008).

The 192.24 Dynamic Array™ by Fluidigm® is a real-time PCR-based, high-throughput SNP genotyping method that offers the possibility of a simplified, resource, and time saving analysis of multiple molecular markers in a single run. Compared to SBE, where every sample and every SNP needs to be tested individually, the Dynamic Array™ enables to analyze a large number of samples and SNPs in parallel. In this study the 192.24 Dynamic Array™ was utilized which analyses up to 24 SNPs and 192 samples. To the best of our knowledge, the 192.24 Dynamic Array™ by Fluidigm® was not yet utilized for the genotyping of ancient DNA samples.

Summing up, the aim of this study was to test whether this high-throughput analysis method which requires only a minimum amount of DNA extract compared to the well-established SBE technique and a considerably reduced data analyses compared to NGS is applicable to ancient and degraded DNA material. The comparison to NGS, however, has to consider that the data set acquired through the SNP array is other than in NGS approaches limited to the investigated SNPs.

Further, the study enabled for the first time to provide information about phenotypic traits of an entire prehistoric human population group of Central Europe. Here, eye and hair colors were determinable for 38 of the 59 investigated 3,000 years old individuals whose skeletal remains were excavated from the Lichtenstein Cave in Lower Saxony, Germany. Through this, the data may contribute to the ongoing scientific debate on the evolutionary history and phenotypes represented in Europe after the replacement of hunter gatherer populations by Neolithic farmers (e.g., Deng & Xu, 2018; Lazaridis, 2018).

2 | CONTAMINATION PREVENTION IN ANCIENT DNA ANALYSES

Due to small numbers of intact target sequences, ancient DNA analyses are principally prone to even spurious contamination. Therefore, a strict regime to prevent contamination prior and in the course of an initial sequence enrichment is mandatory. In the presented study, the first three stages of the in total five-stage analysis are sensitive to contamination events. Therefore, they were carried out in the pre-amplification laboratories of the Department of Historical Anthropology in Göttingen. The laboratories are dedicated to ancient DNA processing only and therefore represent a highly protected environment. The three sensitive stages comprise: (a) the sample preparation consisting of a preventive decontamination of the sample material and grinding it to a fine powder, (b) the DNA extraction consisting of decalcification, cell lysis, and DNA purification, as well as (c) the initial specific target amplification (STA) which is an exponential process leading to an up to a million-fold enrichment of the sequences of interest.

After the initial sensitive stages, the samples were transferred to the Fluidigm® Analysis System which is housed in a modern DNA

laboratory of the Gynaecological Research Unit of the Hannover Medical School. Here, (d) the allele specific amplification for the SNP genotyping was carried out at least four times on aliquots of the enriched samples, and (e) the data analyses.

The processing of the initial stages sensitive to contamination in a specialized ancient DNA laboratory, the multiple allele specific amplifications, the final comparison of the data with those obtained in earlier studies through a SBE approach, which had entirely been carried out in an ancient DNA laboratory, as well as the STR typing of all extracts soundly ensures the authenticity of the results.

3 | SAMPLE DESCRIPTION AND SAMPLE PREPARATION

The samples investigated in this study originate from the human skeletal remains of the Lichtenstein cave. The Lichtenstein cave is located in the Lichtenstein mountain near Osterode in the Harz in Lower Saxony. The cave was discovered in 1972, from 1993 till 2013 a total number of 5,475 human bones of approximately 65 individuals were found (Flindt & Hummel, 2014). The bones were stored since then at −20°C at the department of Historical Anthropology in Göttingen. The bones are dated back to the late Bronze Age and are thus approximately 3,000 years old. Despite the comparatively old age of the skeletal remains particular skeletal elements (e.g., tooth roots, petrosal bones, femurs, and tibias) had already proven to show excellent DNA preservation which allowed at least partial and often even a full STR typing (Burger, Hummel, Herrmann, & Henke, 1999; Flindt et al., 2012; Hummel, Schmidt, Kremeyer, Herrmann, & Oppermann, 2005; Kothe, Seidenberg, Hummel, & Piskurek, 2016; Schultes, Hummel, & Herrmann, 2000; Seidenberg et al., 2012). Most likely this is due to the particularly suitable chemical and physical conditions in the cave. The STR profiles served for kinship analyses and authentification purposes and, over the years in parallel to the ongoing excavation, enabled the reconstruction of an extended family genealogy (Frischalowski et al., n.d.; Schilz, 2006; Schultes et al., 2000; Seidenberg, 2016).

In total DNA extracts of skeletal sample material of 59 individuals were examined with respect to eye and hair color. From the previous research projects (s.a.) DNA extracts of 51 of the individuals were already available, for eight of the individuals which had only been identified from the scattered skeletal material most recently by STR typing (Frischalowski et al., n.d.) DNA extracts were freshly prepared. The DNA extractions were performed following either a DNA concentration using the minElute-filtration by Amicon®, a semiautomated protocol using the Bio Robot EZ1™ of Qiagen® or a protocol using the QiaVac™ system of Qiagen® with optional phenol/chloroform extraction (Frischalowski et al. 2015). The detailed information about the sample preparation including the preventive decontamination of sample material, the different extraction protocols and the different ages of the DNA extracts is found in the Supporting Information (Protocol 1 and Table S1).

4 | SNP TYPING WITH THE 192.24 DYNAMIC ARRAY

For the chip-based SNP genotyping, the 192.24 Dynamic Array™ IFC by Fluidigm® was used in this study. The SNP genotyping consists of an initial target enrichment, the so-called STA which is followed by an allele specific amplification, and a final data analysis consisting of the allele detection and determination. For this, the BioMark™ EP1™ real-time PCR system (Fluidigm Corp., South San Francisco, CA) was used. Suitable SNPs were selected from various papers about FDP (Pneuman, Budimlija, Caragine, Prinz, & Wurmbach, 2012; Söchtig et al., 2015; Walsh et al., 2013) available through an online search using the National Centre for Biotechnology Information database (NCBI; Table S2). Based on these SNPs custom-designed SNPtype™ assays were ordered at the Fluidigm® company. The assays are PCR-based detection systems consisting of a STA primer, a locus-specific reverse primer and an allele-specific primer (ASP). The amplified sequences of the initial target enrichment range between 70 and 129 bp. The ASP is HEX- or FAM-fluorescence labeled to discriminate between both alleles of each SNP. After the ASP amplification the BioMark™ EP1™ Reader measures the fluorescence of each sample, the Fluidigm® SNP Genotyping Analysis™ software calculates the fluorescence signals normalized to a background ROX standard signal. Each sample is then plotted on a scatter plot based on the HEX and FAM relative intensities (HEX on the *X*-axis and FAM on the *X*-axis; Wang et al., 2009). Based on k-means clustering, the genotype of the samples can be determined. The three possible genotypes (two-times homozygosity, one-time heterozygosity) are represented as three clusters in a *X*/*Y*-diagram (see Figure 1), the proximity of a sample to a cluster defines to which genotype the sample is associated.

For the ancient DNA samples, the original Fluidigm® genotyping protocol was modified for some preamplification parameters. In a pretest, it was investigated whether the increase of the DNA extract volume from 1.25 µl to the possible maximum of 2.0 µl would be suitable. Furthermore, the pretest was run with different numbers of cycles in the initial STA phase (14 cycles as suggested by the manufacturer, 20, 25, and 30 cycles). Finally, the pretest used different dilutions of the enriched product during the allele specific amplification (1:100 as suggested by the manufacturer, 1:10 and undiluted). Based on the pretest results the amount of DNA extract volume subjected to the initial STA was increased to the possible maximum of 2 µl. The cycle number of the analysis step was increased to 20 cycles and the dilution of the STA product was decreased to 1:10 for the allele specific amplification step. The pretest results are presented in Figure S1.

The STA, the SNP genotyping as well as the so-called priming, loading, and the thermocycling of the chip followed the protocols as described in the Supporting Information (Protocol 2). In the final step, the data were analyzed with the Fluidigm® SNP Genotyping Analysis™ software and evaluated (cf. Figure S2 [scatter plots]). Each sample was analyzed at least four times. In case of obviously wrong classified samples, the assignment to a cluster was manually corrected (cf. Figure 1).

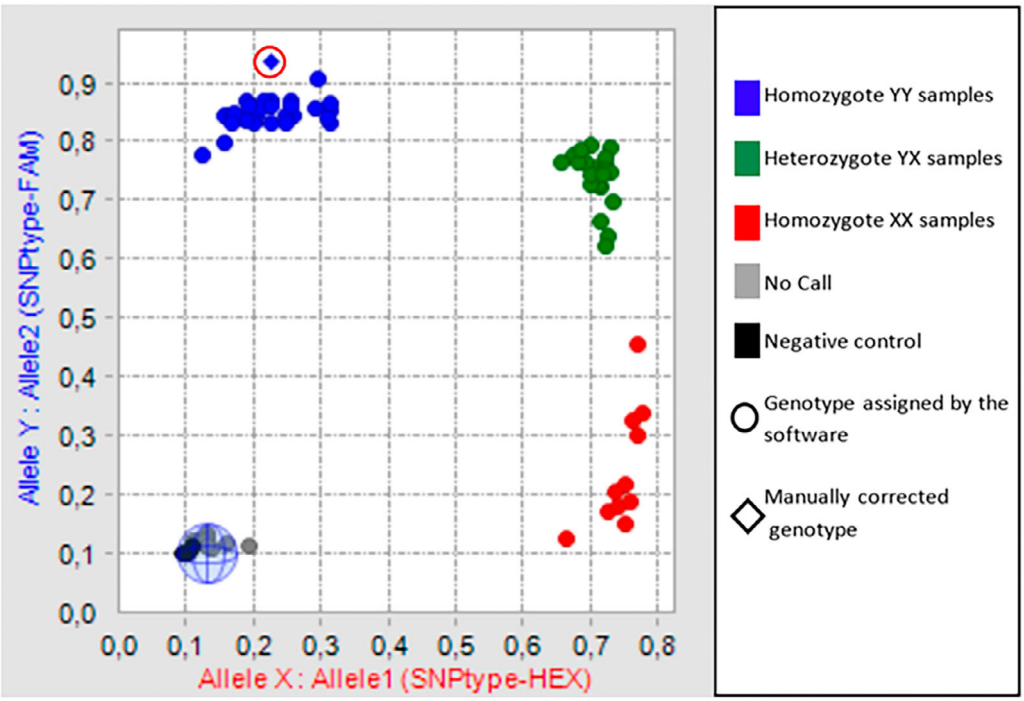

FIGURE 1 The figure shows an exemplary chosen cluster plot obtained with the Fluidigm® analysis software. The two possible alleles for each SNP are named Y and X. In this example, all three possible genotypes (YY, YX, and XX) are presented. Manually corrected data are recognizable by the change of the data point symbol from a dot to a rhombus. An example for this manual correction is here marked with a red circle. In this case, the software had this sample assigned as "No Call," whereas by manual correction it was assigned to the homozygote YY cluster plot

For the phenotyping of the hair and eye color, an online Bayesian classifier termed *Snipper* (http://mathgene.usc.es/snipper/) was used. For the classification of the hair color, the tool named "Classification as individual having fair-dark or red-blond-brown-black hair" and for the eye color the tool named "Classification as individual having blue-green-hazel-brown eyes" was chosen.

Since the SNP chip genotyping method by Fluidigm® utilizes very small volumes of DNA extract, it was intended to verify the achieved results with the genotyping results of the well-established mini-sequencing method (SBE). For 23 of the samples investigated in the presented study SBE genotypes are available. The SBE data for eight SNPs had been processed in earlier studies (Krause, 2012; Schücker, 2012; also cf. supporting information SBE Eye and SBE Hair).

5 | RESULTS

After analyzing all samples and data in the described way, two-thirds (64%) of the ancient DNA samples produced complete or almost complete and reproducible genotypes for all 24 SNPs. Seventeen percent of the samples resulted in partial genotypes with about half of the alleles determined reproducibly. Nineteen percent were classified as failed since single alleles were present only sporadically. The genotypes and most probable phenotypes of all samples are presented in Table 1.

For 68% of the 59 ancient DNA samples, the hair and eye color phenotypes could unambiguously be determined. For 30% of the samples, only tendencies toward one or both phenotypes could be achieved. For 2%, none of the classifying tools could be applied because of a missing genotype. Detailed probabilities of the phenotypes on the basis of the achieved genotypes are presented in Table S3 (hair colors) and Table S4 (eye colors).

The comparison of the two methodological approaches for SNP genotyping using the Fluidigm® Dynamic Array™ and SBE-multiplex typing is presented in Table 2. Full matches between both methodological approaches are present in 90% of the SNP-typing results, 10% reveal nonmatching results. The nonmatching results can either be attributed to be false-homozygote results or a complete analysis failure. The comparison of the phenotypes deriving from the Dynamic Array™ approach and the SBE approaches can be seen in Table S5.

6 | DISCUSSION

The results of our study demonstrate that a genome-wide SNP typing with the Fluidigm® array is possible even for ancient and degraded DNA samples. This allows as many as 24 SNPs typings in parallel for up to 192 samples in a single analysis run. However, the pretesting results of our study show as well that it is mandatory to modify some of the main analysis parameters. Above all, the DNA input must be maximized, the amplification cycles had to be increased, and the STA product serving as a target for the allele-specific amplification was not as much diluted as the manufacturer's instructions suggest. The modifications as well as multiple replications of the analysis are necessary to prevent false-homozygosity. This is due to the fact that degraded

TABLE 1 Genotypes and phenotypes of the Lichtenstein cave samples

Sample (Ind/FN)	rs1129038	rs11538871	rs12203592	rs12821256	rs12896399	rs12913832	rs12931267	rs1393350	rs1545397	rs16891982	rs1800407	rs1805005	rs1805006	rs1805009	rs2228479	rs2378249	rs24021130	rs28777	rs35264875	rs4778138	rs4959270	rs6119471	rs7495174	rs683\ast	Most probable hair color	Most probable eye color
35/107	AG	G	C	T	TG	GA	C	GA	A	G	G	G	C	G	GA	A	A	A	A	A	AC	C	A	-	brown	gh
38/39	A	G	TC	T	T	G	C	G	A	G	G	G	C	G	G	A	A	A	A	A	AC	C	A	-	blond	blue
57.02	AG	G	C	T	TG	(G)A	C	G	A	G	G	G	C	G	A	A	A	A	A	A	A(C)	C	A	-	brown	gh
58.01/65.03	A	G	C	T	T	G	C	GA	A	G	G	G	C	G	G	A	A	A	A	A	AC	C	A	-	blond	blue
58.03	AG	G	TC	T	T	GA	GC	GA	A	G	GA	TG	C	G	G	GA	A	A	TA	A	C	C	A	-	*brown*	gh
58.08/3.01	G	G	C	T	G	A	C	G	A	G	G	T(G)	CA	G	G	A	A	A	A	A	AC	C	A	-	red	brown
65.05	A	-	-	(T)	T	G	G(C)	G	(A)	(G)	G	T(G)	(C)	-	-	-	-	-	A	(A)	-	C	-	-	*blond*	blue
66.02	A	G	C	T	T	G	C	GA	A	G	G	G	C	G	G	A	A	A	A	A	AC	C	A	-	blond	blue
67.03/300.01	GA	G	C	T	TG	GA	C	GA	A	G	(G)	G	C	G	G	A	A	A	TA	A	C	C	A	-	brown	gh
133.01	(GA)	(G)	-	(T)	(T)G	(G)	C	GA	A	G(C)	(G)	T	C	(G)	-	A	A	A	(T)A	(A)	(AC)	(C)	(A)	-	*blond*	gh
183.01	A	G	TC	T	TG	G	C	G	A	G	G	G	C	G	A	(G)	A	A	TA	A	AC	C	A	-	blond	blue
187	A	G	C	-	G	G	C	-	A	-	-	G	C	-	G	G	A	A	A	A	-	(G)C	-	-	blond	blue
516	AG	G	TC	T	TG	GA	C	G	A	G	GA	G	C	G	G	A	A	A	A	A	AC	C	A	-	brown	gh
901.01	A	G	C	T	G	G	C	GA	A	G	G	G	C	G	G	A	A	A	TA	A	C	C	A	-	blond	blue
902.01/480.01	AG	G	TC	TC	T	GA	C	G	A	G	G	G	C	G	GA	A	A	A	A	A	AC	C	A	-	brown	*gh*
903.01	AG	G	C	T	G	GA	C	GA	A	G	G	TG	C	G	G	A	G	A	TA	GA	AC	C	A	-	brown	gh
903.02/58.04	AG	G	C	T	G	GA	C	GA	A	G	G	TG	C	G	G	GA	A	A	TA	A	C	C	A	-	brown	gh
904.05	-	-	-	-	-	-	-	-	-	-	-	T	C	-	(G)	-	-	-	-	-	-	-	-	-	*[brown]*	–
905.01	A	G	C	T	G	G	C	GA	A	G	G	G	C	G	GA	A	G	A	TA	A	C	C	A	-	blond	blue
907.01	G	G	T	T	TG	A	C	GA	A	G	(GA)	G	C	G	G	A	G	A	TA	GA	-	C	A	-	*black*	brown
1076	AG	G	C	T	G	GA	C	G	TA	G	GA	TG	C	G	G	A	GA	A	A	A	C	C	A	-	brown	gh
1078/1044	AG	G	C	T	G	GA	C	G	A	G	GA	G	C	G	G	A	GA	A	A	A	C	C	A	-	brown	gh
1102	G	-	C	-	T	GA	C	G	A	G	G	TG	C	G	G	(A)	(A)	A	A	-	A	C	A	-	brown	*brown*
1103	(AG)	(G)	(C)	(T)	(G)	(GA)	(GC)	-	(A)	(G)	(GA)	G	(C)	(G)	-	A	(A)	(A)	(A)	(A)	(A)	(C)	(A)	-	red	gh
1176/2133	A	G	C	T	G	G	GC	G	A	G	G	G	C	G	G	GA	GA	A	TA	A	AC	C	A	-	blond	blue
1247/1745.1	A	G	C	T	TG	G	C	GA	A	G	G	G	C	G	G	A	GA	A	A	A	AC	C	A	-	blond	blue
1461	A	G	T	(T)	-	G	C	G	A	(G)	G	T	C	(G)	G	A	A	A	A	A	(AC)	C	(A)	-	blond	blue
1467.01	-	(G)	-	-	-	-	G	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	*[red]*	–
1471	A	G	C	T	TG	G	C	G	TA	G	GA	G	C	G	G	A	A	A	A	A	C	C	A	-	blond	blue
1482/1183	AG	G	TC	T	T(G)	(G)A	C	G	A	G	G	TG	C	G	G	A	GA	A	A	(G)A	A	C	A	-	brown	gh

(Continues)

TABLE 1 (Continued)

Sample (Ind/FN)	rs1129038	rs11538871	rs12203592	rs12821256	rs12896399	rs12913832	rs12931267	rs1393350	rs1545397	rs16891982	rs1800407	rs1805005	rs1805006	rs1805009	rs2228479	rs2378249	rs24021130	rs28777	rs35264875	rs4778138	rs4959270	rs6119471	rs7495174	rs683\ast	Most probable hair color	Most probable eye color
1500/1827.01	A	G	TC	T	TG	G	C	G	A	GC	G	G	C	G	G	A	A	A	A	A	AC	C	A	-	**blond**	**blue**
1547.01	(A)	-	-	-	-	-	(G)	-	-	-	-	T	C	-	G	-	-	-	-	-	-	-	A	-	**[red]**	–
1547.03	A	G	C	(T)	T	G	C	G	T	G	G	G	C	G	G	A	A	A	A	A	C	C	A	-	**blond**	**blue**
1,585.03	A	G	C	T	TG	G	C	G	A	G	G	G	C	G	GA	GA	A	A	A	A	C	C	A	-	**blond**	**blue**
1745.02	A	G	C	T	TG	G	C	G	A	G	G	TG	C	G	G	A	A	A	A	A	C	C	A	-	**blond**	**blue**
1905/1917	AG	G	C	T	TG	GA	C	G	A	G	G	G(T)	C	G	G	GA	A	A	TA	GA	AC	C	A	-	**brown**	**gh**
2030.01	AG	G	TC	T	T	GA	C	G	A	G	G	G	C	G	G	A	A	A	A	GA	AC	C	GA	-	**brown**	***gh***
2263	(A)	(G)	(C)	(T)	(TG)	(G)	(C)	(G)	(A)	-	(G)	T	(C)	(G)	-	A	(A)	A	A	(A)	C	-	(A)	-	**blond**	**blue**
2588	A	G	C	T	TG	G	C	G(A)	A	GC	G	G	C	G	G	GA	A	CA	A	A	C	C	A	-	***black***	**blue**
3190.11	G	G	C	T	G	A	C	G	A	G	GA	G	C	G	G	A	G	A	A	A	C	(C)	A	-	***black***	**brown**
3628/1746.06	AG	G	C	T	G	A	C	G	A	G	GA	G	C	G	G	A	-	A	A	-	C	-	A	-	***black***	**brown**
3695/4078	AG	G	C	T	TG	GA	C	GA	A	G	G	TG	C	G	G	A	A	A	TA	GA	AC	C	A	-	**brown**	**gh**
3706/3756	G	G	C	T	TG	A	C	GA	A	G	GA	G	C	G	G	A	A	A	A	GA	C	C	A	-	***black***	**brown**
3714	(G)	(G)	-	(T)	(G)	-	(G)	(G)	-	(G)	-	T	C	(G)	G	-	-	(A)	-	-	-	-	-	-	***[brown]***	–
3742/4460	AG	G	C	T	G	GA	C	G	A	G	G	G	C	G	A	A	A	A	TA	A	C	C	A	-	**brown**	**gh**
3748/3631	G	G	(T)	T	-	A	C	GA	A	-	G	T(G)	-	G	-	A	A	A	-	A(G)	-	C	-	-	***black***	**brown**
3757	GA	G	TC	T	TG	GA	C	GA	A	G	G	G	C	G	G	A	A	A	A	GA	AC	C	A	-	**brown**	**gh**
3886	G	G	TC	T	TG	A	C	GA	TA	G	G	G	C	G	G	A	A	A	A	GA	AC	C	GA	-	**black**	**brown**
4008/2096	A	G	C	T	T	G	C	GA	A	G	G	G	C	G	GA	A	A	A	A	A	AC	C	A	-	**blond**	**blue**
4310.01/3812	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	**–**	–
5517	G	G	T(C)	T	G	A	C	G	A	G	GA	G	C	G	G	A	A	A	A	GA	AC	C	A	-	***black***	**brown**
7043.01/7339	A	G	C	T	G	G	C	GA	A	G	G	G	C	G	GA	GA	A	A	A	A	AC	C	A	-	**blond**	**blue**
7075.01	A	G	C	T	TG	G	C	GA	A	G	G	TG	C	G	G	A	A	A	A	A	AC	C	A	-	**blond**	**blue**
E11	A	(G)	C	(T)	-	G	C	G	-	(G)	(G)	T	C	G	G	A	A	A	(A)	A	(C)	-	-	-	**blond**	**blue**
E33	A	(G)	-	-	-	-	C	-	-	G	-	TG	C	G	G	(A)	(A)	(A)	-	-	(C)	-	-	-	**blond**	**[blue]**
E37	AG	G	TC	(T)	T	GA	C	GA	-	G	GA	T	C	(G)	G	GA	(A)	A	(T)	A	(A)	C	GA	-	**brown**	**gh**
N957.01	-	-	T	-	(G)	(G)	C	-	A	G	G	GT	C	(G)	G	A	-	A	A	A	A	C	(GA)	-	**blond**	**blue**
N962.01	-	G	-	-	-	-	(G)	-	-	-	-	TG	(C)	(C)	-	G(A)	-	-	-	-	-	-	(A)	-	**[red]**	–
R1	AG	G	C	T	G	GA	C	G	TA	G	G	G	C	G	G	A	A	A	T	A	C	C	A	-	**brown**	***gh***

Notes: Ind = Individual, FN = Finding number, – = no call, written in *italic* = not classified (under 3:1 ratio Snipper), written in bold = highest probability, written in square brackets [] = based on small number of SNPs, gh = green-hazel, - = no prediction possible because of a missing genotype; *SNP rs683 was excluded from further analysis.

TABLE 2 Comparison of genotyping results based on the Dynamic Array™ and SBE of 23 Lichtenstein cave samples

Individual	Finding number	Method	rs12203592	rs12896399	rs12913832	rs1393350	rs16891982	rs1800407	rs1805005	rs28777
	39	Dynamic Array™	TC	T	G	G	G	G	G	A
38	39	SBE_Eye	TC	TG	G	G	G	G		
	39	SBE_Hair		TG	G	G	G		G	A
	57.02	Dynamic Array™	C	TG	A(G)	G	G	G	G	A
57.02	57.02	SBE_Eye	C	TG	AG	G	G	G		
	57.02	SBE_Hair		TG	AG	G	G		G	A
	65.01	Dynamic Array™	CT	T	GA	GA	G	GA	(G)T	A
58.03	58.03	SBE_Eye	C	T	GA	GA	G	GA		
	58.03	SBE_Hair		T	GA	GA	G		GT	A
	901.01	Dynamic Array™	C	G	G	GA	G	G	G	A
901.01	901.01	SBE_Eye	C	G	G	G	G	G		
	901.01	SBE_Hair		G	G	G	G		G	A
	480.01	Dynamic Array™	TC	T	GA	G	G	G	G	A
902.01	902.01	SBE_Eye	TC	T	GA	G	G	G		
	902.01	SBE_Hair		T	GA	G	G		G	A
	1076	Dynamic Array™	C	G	GA	G	G	GA	TG	A
1076	1076	SBE_Eye	C	G	GA	G	G	GA		
	1076	SBE_Hair		G	GA	G	G		TG	A
	1044	Dynamic Array™	C	G	GA	G	G	GA	G	A
1078	1044	SBE_Eye	C	G	GA	G	G	GA		
	1044	SBE_Hair		G	GA	G	G		G	A
	1102	Dynamic Array™	C	T	GA	G	G	G	TG	A
1102	3670	SBE_Eye	TC	GT	GA	G	G	AG		
	3670	SBE_Hair		GT	GA	G	G		(T)G	A
	1103	Dynamic Array™	(C)	(G)	(GA)	X	(G)	(GA)	G	(A)
1103	1103	SBE_Eye	C	G	GA	G	G	GA		
	1103	SBE_Hair		G	GA	G	G		G	A
	2133	Dynamic Array™	C	G	G	G	G	G	G	A
1176	199	SBE_Eye	C(T)	G	G	G	G	G		
	199	SBE_Hair		G	G	G	G		G	A
	1745.1	Dynamic Array™	C	TG	G	GA	G	G	G	A
1247	6495	SBE_Eye	C	TG	G(A)	GA	G	G		
	6495	SBE_Hair		TG	G	GA	G		G	A

(Continues)

TABLE 2 (Continued)

Individual	Finding number	Method	rs12203592	rs12896399	rs12913832	rs1393350	rs16891982	rs1800407	rs1805005	rs28777
	1183	Dynamic Array™	TC	TG	AG	G	G	G	TG	A
1482	1911	SBE_Eye	TC	TG	AG	G	G	G		
	1911	SBE_Hair		TG	AG	G	G		TG	A
	1827.01	Dynamic Array™	TC	TG	G	G	GC	G	G	A
1500	1827.01	SBE_Eye	TC	TG	G	G	GC	G		
	1827.01	SBE_Hair		TG	G	G	GC		G	A
	1585.03	Dynamic Array™	C	TG	G	G	G	G	G	A
1585.03 (DII/III)	1585.03	SBE_Eye	C	TG	G	G	G	G		
	1585.03	SBE_Hair		TG	G	G	G		G	A
	1917	Dynamic Array™	C	TG	GA	G	G	G	GT	A
1905	1917	SBE_Eye	C	TG	GA	G	G	G		
	1917	SBE_Hair		TG	GA	G	G		GT	A
	2030.01	Dynamic Array™	TC	T	GA	G	G	G	G	A
2030.01	2030.01	SBE_Eye	TC	T	GA	G	G	G		
	2030.01	SBE_Hair		T	GA	G	G		G	A
	2588	Dynamic Array™	C	TG	G	G(A)	GC	G	G	CA
2588 (DOI)	2588	SBE_Eye	C	TG	G	G	GC	G		
	2588	SBE_Hair		TG	G	G	GC		G	CA
	3756	Dynamic Array	C	TG	A	GA	G	GA	G	A
3706	3756	SBE_Eye	C	TG	A	GA	G	GA		
	3756	SBE_Hair		TG	A	GA	G		G	A
	3714	Dynamic Array™	X	(G)	X	(G)	(G)	X	T	(A)
3714	2096	SBE_Eye	C	TG	GA	G	G	G(A)		
	2096	SBE_Hair		TG	GA	GA	G		G	A
	4460	Dynamic Array™	C	G	GA	G	G	G	G	A
3742	3742	SBE_Eye	C	G	GA	G	G	G		
	3742	SBE_Hair		G	GA	G	G		G	A
	3631	Dynamic Array™	T	X	A	GA	X	G	(G)T	A
3748	5521	SBE Eye	TC	(G)T	GA	GA	G	G		
	5521	SBE_Hair		T	GA	GA	G		GT	A
	2096	Dynamic Array™	C	T	G	GA	G	G	G	A
4008	2096	SBE_Eye	C	T	G	GA	G	G		
	2096	SBE_Hair		T	G	GA	G		G	A

(Continues)

TABLE 2 (Continued)

Individual	Finding number	Method	rs12203592	rs12896399	rs12913832	rs1393350	rs16891982	rs1800407	rs1805005	rs28777
	5517	Dynamic Array™	TC	G	A	G	G	GA	G	A
5517	3710	SBE_Eye	TC	G	A	G	G	GA		
	3710	SBE_Hair		G	A	G	G		G	A

Notes: Dynamic Array™ = 192.24 Dynamic Array™ IFC genotyping results, SBE_Eye = Single base extension genotyping results of Krause (2012), SBE_Hair = Single base extension genotyping results of Schücker (2012), X = no genotyping result.

DNA from archeological skeletal material shows characteristics which may impede the analysis. These are DNA fragmentation and thus only small amounts of intact target DNA, microbial DNA from soil present in the extract, as well as remaining inhibiting substances of different kind. Therefore, a typical problem when analyzing aDNA is the difficulty in reaching the detection limit resulting in allelic dropout or seemingly complete amplification failures. In case of an allelic dropout, an imbalance in the presence of intact target sequences leads to an unequal amplification result causing a false homozygosity (Butler & Hill, 2010; Hummel, 2003). However, a false homozygosity may also be caused through mismatch in one of the primer hybridization sites which is known as so-called null alleles. This artifact cannot be identified by repeated amplification analysis, only the use of different primers can overcome the generation of false-homozygous result. This is due to the fact that a mutation in the primer binding region of one of the alleles prevents the amplification of the affected allele, thus causing a false homozygosity (Butler, 2001).

The genotypes based on the Fluidigm® 192.24 Dynamic Array™ were compared with the genotypes based on the well-established SBE analyses (Table 2). The SBE data were derived from two previous studies which carried out a genotyping for 23 samples and eight of the SNPs in the presented study. The comparison shows that a large majority of results fully match, while again the majority of the non-matching results are represented through those cases, where the SBE approach resulted in a heterozygote genotype whereas the Dynamic Array™ approach shows a homozygote result. This may most likely be explained by allelic dropout events in the Fluidigm® SNP chip approach. Since allelic drop out results from imbalanced intact target situations, it can best be prevented by enlarged DNA input already to the initial STA. To achieve this for the Fluidigm® analysis system, an increase of the DNA concentration should be aimed, because the 2 μl DNA input as carried out in the study already represents the maximum volume for the initial amplification step. As well, the number of amplification cycles may further be enlarged. Following the results of our pretesting the increase to 20 cycles was sufficient for the Lichtenstein cave samples. However, DNA extracts with lesser DNA contents may benefit from more cycles. At least in our set of samples, larger number of amplification cycles did apparently not cause harm to the results as can be derived from the pretesting results.

In one sample (901.01), the Dynamic Array™ approach resulted in a soundly reproduced heterozygote genotype for one of the SNPs (rs1393350), in contrast the even so reproduced SBE approaches resulted in a homozygote genotype for this SNP. In a second sample basically the same applies, however the SNP in focus (rs12203592) had been part of one SBE approach (eye color) only. In a third sample (2588 DOI), the results of the Fluidigm® Dynamic Array™ were contradicting with respect to heterozygosity which is why the A-allele was kept in brackets. Therefore, the following discussion is restricted to sample 901.01. In general, allelic dropout could also serve as an explanation for a false homozygosity of SBE results. However, in this particular case, the occurrence of a so-called null allele would be more plausible, since the heterozygous result occurred in the Fluidigm® approach which is based on a lesser amount of DNA extract compared

to the SBE approaches. Therefore, if any, it would be the Fluidigm® approach to be considered to be more prone to allelic dropout and thus the false homozygosity.

Another possible source of deviating results, particularly in the just discussed samples, is the genotype call with the Fluidigm® analysis software. In the respective cases (58.03, 901.01, and 2588 DOI), the allocation of samples to the cluster plots (Figure S2) are not distinct. The samples were determined as heterozygote with the Dynamic Array™ but homozygote with the SBE method. Examining the individual cluster plots reveals that in these cases, the dots marked as heterozygote are located either close to the homozygote cluster or in the middle between the two clusters. The proximity of a sample to a cluster defines which genotype is attributed to the sample. Mistakenly attributed genotypes may be prevented through manual correction of ambiguous results. However, experience with characteristics of the respective scatter plot pattern is mandatory.

Summing up, the study could show that the Fluidigm® Dynamic Array™ is suitable for high-throughput SNP typing. The high genotyping success rate was achieved by modifications of the preamplification protocol mainly by increasing the amount of DNA extract in the initial amplification step and the increase of amplification cycles. The occurrence of allelic dropouts indicates that a further increase of DNA input to the preamplification step would be desirable, particularly if less well-preserved ancient DNAs as the ones from the Lichtenstein cave are investigated.

With the applied technique, it was for the first time possible to get information about major phenotypic traits—eye and hair color—of an entire prehistoric population. The range of traits, varying from blonde to brown hair and blue to green-hazel eye colors for the majority of individuals is a plausible result for a Central European population.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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