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TECHNICAL NOTE

CRIMINALISTICS

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Forensic SNP Genotyping with SNaPshot: Development of a Novel In-house SBE Multiplex SNP Assay*,†

ABSTRACT: This study introduces a newly developed in-house SNaPshot single-base extension (SBE) multiplex assay for forensic single nucleotide polymorphism (SNP) genotyping of fresh and degraded samples. The assay was validated with fresh blood samples from four different populations. In addition, altogether 24 samples from skeletal remains were analyzed with the multiplex. Full SNP profiles could be obtained from 14 specimens, while ten remains showed partial SNP profiles. Minor allele frequencies (MAF) of bone samples and different populations were compared and used for association of skeletal remains with a certain population. The results reveal that the SNPs of the bone samples are genetically close to the Pathan population. The findings show that the new multiplex system can be utilized for SNP genotyping of degraded and forensic relevant skeletal material, enabling to provide additional investigative leads in criminal cases.

KEYWORDS: forensic science, forensic genetics, forensic genotyping, skeletal remains, multiplex development, single-base extension, population genetics, single nucleotide polymorphism

Skeletal remains are able to preserve DNA for a long time (1). For that reason, they are often utilized for ancient archeological or anthropological DNA analysis or for identification of missing persons in, for example, mass disasters in forensic genetics (2–4). Mostly, short tandem repeats (STRs) are used for DNA typing. However, highly degraded bone samples frequently contain inhibitors as well as low quantities of DNA. Tannin, humic acid, fulvic acid, polymerase inhibitors, hydroxylapatite, collagen type 1, and maillard products are the main inhibitors that can prevent DNA amplification of those samples (5–7). Due to the presence of stochastic effects such as allelic/locus drop-outs, allelic/locus drop-ins, heterozygote peak imbalance, and reduced allelic peak heights, the interpretation of DNA profiles from skeletal remains can be very difficult (8–12). In cases of highly degraded DNA samples, STR typing often fails to produce full DNA profiles (13); therefore, shorter markers like mini-STRs and single nucleotide polymorphisms (SNPs) can be used additionally to get investigative leads in cases where there is no database or suspect match (14,15).

Although applications that use very short amplicons such as the SNaPshot approach are preferred for genotyping of highly degraded DNA, the SNaPshot approach faces challenges such as primer design, PCR optimization, single-base extension (SBE) chemistries, and interpretation of the peak patterns observed in typical forensic SNP profiles (16). SNP genotyping is an emerging field in forensic sciences, which allows the identification of highly degraded biological samples by analyzing genetic information of the donor's samples (17–19). The SNP genotyping of degraded DNA material is expected to be useful in forensic investigations as it may provide additional genetic information helpful for the identification of unknown skeletal remains. Genetic information from skeletal remains can be determined on the basis of SNPs. Hereby, we introduce an in-house SNaPshot SBE multiplex system for forensic SNP genotyping, which can be used for geographical assignment of

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†All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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both fresh and highly degraded DNA samples including skeletal remains.

Materials and Methods

Collection of Samples

Bone samples: 24 bone samples were collected randomly from 200- to 500-year-old mass graves of the Khyber Pakhtunkhwa province of Pakistan. Approval for the collection of bone samples was attained from the Ethical Committee of the National Centre of Excellence in Molecular Biology University of the Punjab Lahore Pakistan. Bone samples were labeled, photo-documented, and stored at -20°C until analysis.

Blood samples: Blood samples from 80 unrelated healthy individuals of Korean, African American, Caucasian, and Pathan (Pakistani Pathan of the Khyber Pakhtunkhwa) populations were collected with informed consents that were approved by the Ethical Committee of the National Centre of Excellence in Molecular Biology University of the Punjab Lahore Pakistan and the Forensic Medicine Department of Yonsei University College of Medicine South Korea. Three millilitre of blood was drawn from each individual by venipuncture using evacuated or vacuum tubes (with anticoagulants such as EDTA to prevent clotting) for blood collection. The blood samples were stored at 4°C and processed immediately. Storage for long-term use was in aliquots at −70°C.

Preparation of Bone Samples

The bone samples were divided into small fragments using a dental diamond disk and exposed to ultraviolet (UV) light for 30 min. The bone fragments were scrubbed with Dremel tools and surgical scalpel blades, treated with bleach (10%), ddH2O and ethanol (95%) to remove latent contaminations and placed under a sterilized fume hood overnight. The samples were grinded into fine powder using liquid nitrogen and a SPEX 6750 Freezer/Mill (SPEX CertiPrep, Metuchen, NJ). The powders were shifted to 15-mL falcon tubes and kept at -20°C until DNA extraction. 0.5 g bone powder per sample was used for DNA extraction.

DNA Extraction and DNA Quantification

The DNA of the blood samples was extracted by organic phenol–chloroform extraction (20). DNA extraction from the bone samples was performed by a modified silica column-based total demineralization extraction method according to the protocol reported in the manuscript of Zar et al. (21). The Quantifiler® Human Duo DNA Quantification Kit was used for DNA quantification, following the instructions of the manufacturer's protocol (Applied Biosystems, Foster City, USA). DNA was quantified in replicates.

SNPs Amplification

The in-house SNaPshot SBE multiplex system consists of nine SNPs: rs885479 (MC1R), rs26722 (SLC45A2), rs2031526 (DCT), rs7495174 (OCA2), rs4778241 (OCA2), rs4778138 (OCA2), rs1800414 (OCA2), rs1545397 (OCA2), and rs12913832 (HERC2) (details and primer sequences of all markers in Tables 1 and 2). Amplification of genomic DNA was carried out with a multiplex PCR in a volume of 10 μL for

TABLE 1—*PCR Primers used for the amplification of the SNaPshot SBE markers.*

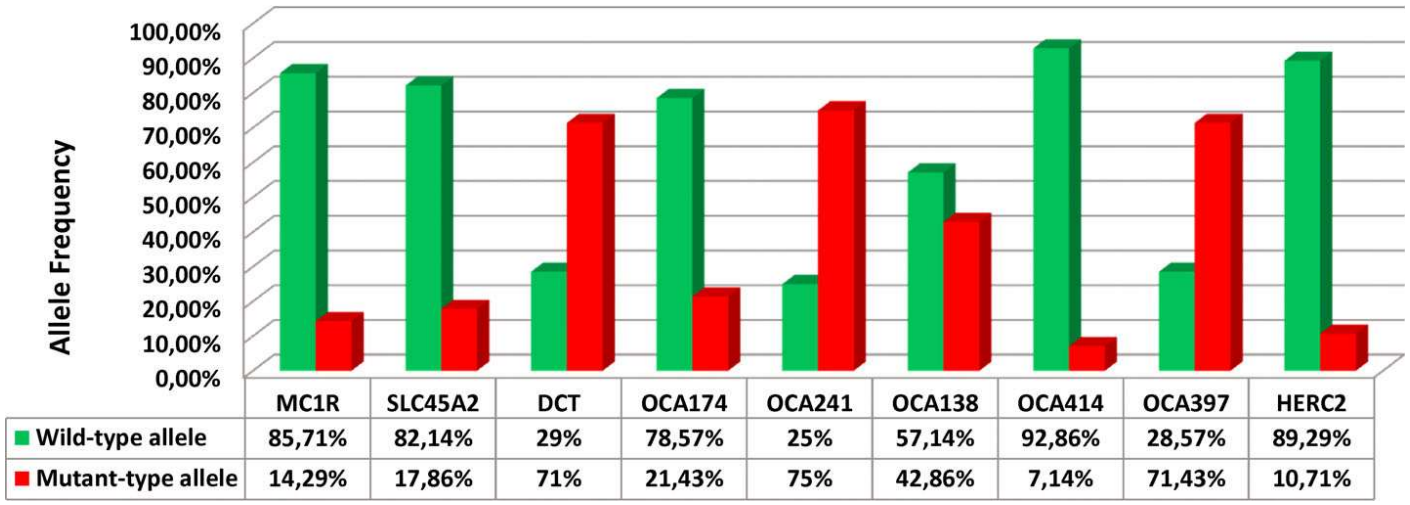
Gene	Reference SNP ID*	SNV† (Alleles)	Phenotype	Location	Protein	Primers	Sequence (5′-3′)	Primer length (bp)	Concentration (μM)	Amplicon size (bp)
MC1R	rs885479	A/G	Skin color	16q24.3	Melanocortin 1 receptor	Forward	GTG GAC CGC TAC ATC TCC AT	20	0.3	119
						Reverse	AAG AGC GTG CTG AAG ACG AC	20	0.3	
SLC45A2	rs26722	C/T	Hair color,Skin color	5p13.3	Membrane-associated transporter protein	Forward	CAG GAC CCT CCA TTG TCA TC	20	0.25	134
						Reverse	TGC ATC TTT ACC TGT TCA GCA	21	0.25	
DCT	rs2031526	A/G	Skin color	13q32	Dopachrome tautomerase	Forward	CCT TGA ATT GCT CTT GAA AAA CTA A	25	0.8	149
						Reverse	CAG CCC AAT GAT ACA CTT TCA TTT AAC	27	0.8	
OCA2	rs7495174	A/G	Eye color	15q11.2-15q12	NA+/H+ antiporter or glutamate transporter	Forward	AGG CCC AGG CGG ACT CAG	18	0.6	128
						Reverse	AGG CAG GGA GGG TTT ACA CAG C	22	0.6	
OCA2	rs4778241	A/C	Eye color			Forward	GCC ACT CTG GAA AGC AGT TT	20	0.5	133
						Reverse	CCA TTT GCG TGT AGG GTT TT	20	0.5	
OCA2	rs4778138	A/G	Eye color			Forward	GCT GTA AAT TTC CTC CCA TCA C	22	0.8	116
						Reverse	TCA AAA AGA AAG TCT CAA GGG AA	23	0.8	
OCA2	rs1800414	A/G	Eye color, Skin color			Forward	TCG TGA TTC CAG TTG CGT AG	20	0.25	135
						Reverse	CCA ACA CTG TCA GGC ATT TG	20	0.25	
OCA2	rs1545397	A/T	Eye color			Forward	TGG AAT TGG ATA CTG ACA ATG GTT G	25	1.0	144
						Reverse	CAT GGG GGA GAG AGA ATG ACT CAG	24	1.0	
HERC2	rs12913832	A/G	Eye color	15q13	E3 ubiquitin protein ligase HERC2	Forward	TTG TTC TTC ATG GCT CTC TGT GTC TG	26	0.5	108
						Reverse	AGA GAA GCC TCG GCC CCT GA	20	0.5	

*"Reference SNP ID" refers to the reference sequence identifier given to the SNP in the dbSNP database.
†"SNV" stands for single nucleotide variation.

TABLE 2—*Minisequencing primers used for the detection of the SNaPshot SBE markers.*

Gene	Reference SNP ID	Primer Direction	Primer Sequence (5′–3′) with t-tail	Primer Length Without t-tail (bp)	Total Primer Length (bp)	Concentration (μM)
MC1R	rs885479	Reverse (R19-20)	tCC AGA TGG CCG CAA CGG CT	19	20	0.8
SLC45A2	rs26722	Forward (F23-25)	ttG AAT GTA CGA GTA TGG TTC TAT C	23	25	0.15
DCT	rs2031526	Reverse (R22-31)	ttt ttt ttt AAA TGT CAT TTG AGG GTA GGA A	22	31	1.0
OCA-174	rs7495174	Reverse (R21-38)	ttt ttt ttt ttt ttt ttA AGG CAA GTT CCC CTA AAG GT	21	38	0.2
OCA-241	rs4778241	Reverse (R19-44)	ttt ttt ttt ttt ttt ttt ttt ttt tTT GGC TGG TAG TTG CAA TT	19	44	0.3
OCA-138	rs4778138	Forward (F24-50)	ttt ttt ttt ttt ttt ttt ttt ttt ttC ATC ACT GAT TTA GCT GTG TTC TG	24	50	0.5
OCA-414	rs1800414	Forward (F21-57)	ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt CTG TGG TTT CTC TCT TAC AGC	21	57	0.15
OCA-397	rs1545397	Forward (F29-63)	ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt tAA TTT ATC TTG CAA AAT TAT ATC ATT CAG	29	63	2.0
HERC2	rs12913832	Reverse (R19-68)	ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt ttt tTA GCG TGC AGA ACT TGA CA	19	68	0.15

samples with DNA concentrations above 10 pg/μL and 20 μL for samples below 10 pg/μL. PCR input of DNA extracts for samples with over 100 pg/μL was 1 μL and for samples between 10 and 100 pg/μL 2 μL. 10 μL of DNA was used for samples below 10 pg/μL. Final reaction volume of 10 μL contained 2.0 μL 5× primer mix, 0.6 μL (5 U/μL) AmpliTaq



	MC1R	SLC45A2	DCT	OCA174	OCA241	OCA138	OCA414	OCA397	HERC2
Wild-type allele	85,71%	82,14%	29%	78,57%	25%	57,14%	92,86%	28,57%	89,29%
Mutant-type allele	14,29%	17,86%	71%	21,43%	75%	42,86%	7,14%	71,43%	10,71%

FIG. 1—*Wild-type and mutant-type allele frequencies of the nine SNPs across skeletal remains.*

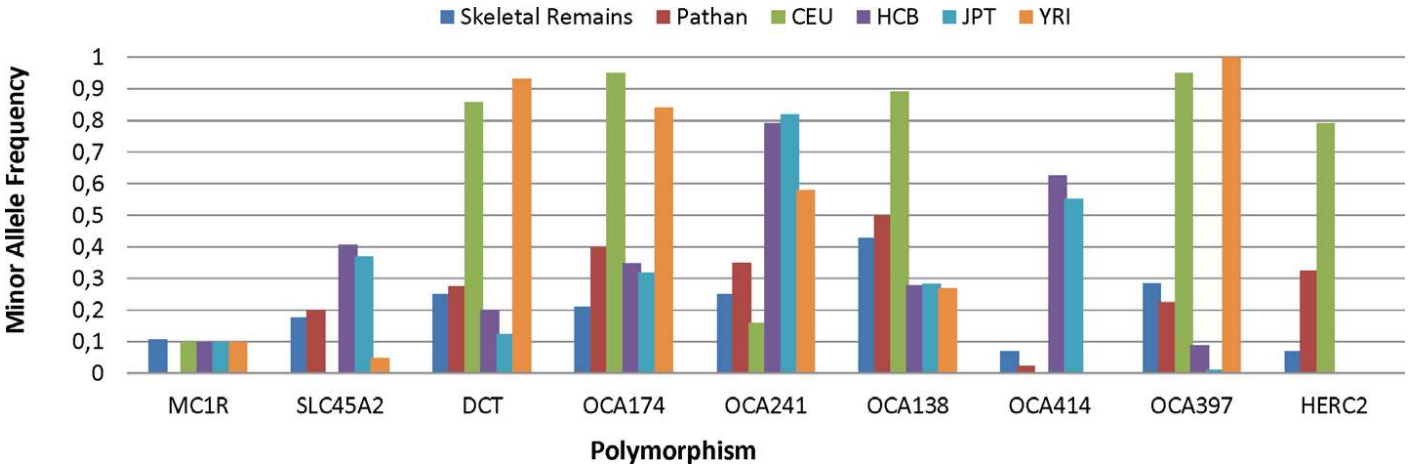


FIG. 2—*Association of minor allele frequencies (MAF) of the nine SNPs across skeletal remains and other populations.*

Gold® DNA Polymerase (ABI), 1.0 μL 10× Gold STR buffer, and either 4.4 μL or 5.4 μL dH_2O. 20 μL reaction volumes consisted of 4.0 μL 5× primer mix, 0.8 μL (5 U/μL) AmpliTaq Gold® DNA Polymerase (ABI), 2.0 μL 10× Gold STR buffer, and 3.2 μL dH_2O. Thermal cycling was conducted on a PTC-200 DNA engine cycler (MJ Research, USA) using the following PCR conditions: 95°C for 11 min, 94°C for 20 sec, 60°C for 1 min, 72°C for 30 sec, and a final extension at 72°C for 7 min. The number of PCR cycles was 38 during all experiments. To remove the remaining dNTPs and primers from the PCR products, 1.5 μL exonuclease and shrimp alkaline phosphatase (Exo-SAP-IT) were added to 5 μL of PCR product and treated for 45 min at 37°C and for 15 min at 80°C. 1.0 μL of Exo-SAP-treated PCR product was mixed with 2.0 μL 5× sequencing buffer (Thermo Fisher Scientific), 1.0 μL SNaPshot reaction mix (Thermo Fisher Scientific), 2 μL 5× primer mix, and 4.0 μL dH_2O in a final reaction volume of 10 μL to conduct the SBE multiplex reaction at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 30 sec with 25 PCR cycles. Unincorporated fluorescent ddNTPs were removed from SBE PCR products by treatment with 1.5 μL shrimp alkaline phosphatase (SAP) at 37°C for 45 min and 80°C for 15 min. All samples were genotyped in replicates, and consensus profiles were generated for each bone. Negative controls using nuclease-free water were run with all PCR amplification reactions.

Capillary Electrophoresis

The samples were prepared for capillary electrophoresis by loading 10 μL Hi-Di formamide (ABI), 1.0 μL SBE PCR product of each sample and 0.2 μL GeneScan® 120-LIZ™ size standard. The samples were heat-shocked at 95°C for 5 min to denature DNA into single-stranded DNA, immediately placed on ice for 3 min and then run on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Data Analysis

The data were analyzed with the GeneMapper ID software version 3.2 (Applied Biosystems, Foster City, USA). The chi-square Hardy–Weinberg equilibrium test calculator was used for the analysis of the allele frequencies of all SNPs (22). *P*-values were determined with the HWE calculator of Michael H. Court (https://www.researchgate.net/file.PostFileLoader.html?id=58dbf27a96b7e4fc194da329&assetKey=AS%3A477303651213312%401490809466020).

Results and Discussion

In this study, 9 SNPs were selected to create a SNaPshot SBE multiplex assay. The in-house SNaPshot SBE multiplex system consists of SNPs of the HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2), oculocutaneous albinism II (OCA2), melanocortin 1 receptor (MCR1), solute carrier family 45 member 2 (SLC45A2), and dopachrome tautomerase (DCT) genes, which play significant roles in SNP genotyping. All of those genes are involved in the mammalian skin, hair, and eye pigmentation systems and are already forensically used for phenotyping (23–26). All SNPs included into the assay are biallelic [wild type (W) and mutant type (Mt)]. SNP analysis can be used for the detection of DNA polymorphisms among different populations (27). To confirm the authenticity of this multiplex system, 80 blood samples from four different populations including

TABLE 3—*Analysis of the allele frequencies of all 9 SNPs across skeletal remains and other populations.*

Information about SNPs			Skeletal Remains from Pakistan					HapMap				Concordance and Discordance	Reference
SNP ID	Reported gene	Phenotype	Major allele (A)	Minor allele (B)	MAF	HWE*	No. of genotypes (AA/AB/BB)	*P*-value	Pathan	CEU	HCB	JPT	YRI		
rs885479	*MC1R*	Skin color	G	A	0.107	5.5	(12/1/1)	0.019	0.00	0.100	0.640	0.733	0.009	Major in HCB & JPT	(38)
rs26722	*SLC45A2*	Hair color Skin color	C	T	0.178	1.02	(10/3/1)	0.313	0.20	0.004	0.407	0.372	0.049	Minor in all	(30)
rs2031526	*DCT*	Skin color	A	G	0.25	9.17	(10/1/3)	0.002	0.275	0.858	0.200	0.125	0.933	Major in CEU & YRI	(39)
rs7495174	*OCA2*	Eye color	G	A	0.21	4.64	(10/2/2)	0.031	0.40	0.951	0.349	0.320	0.841	Major in CEU & YRI	(40)
rs4778241		Eye color	C	A	0.25	9.17	(10/1/3)	0.002	0.35	0.159	0.791	0.820	0.580	Major in HCB, JPT & YRI	(41)
rs4778138		Eye color	G	A	0.428	0.22	(5/6/3)	0.639	0.50	0.894	0.279	0.283	0.270	Major in CEU only	(42)
rs1800414		Eye color Skin color	A	G	0.071	14	(13/0/1)	0.0002	0.025	0.00	0.628	0.552	0.00	Major in HCB & JPT	(39)
rs1545397		Eye color	T	A	0.285	5.92	(9/2/3)	0.015	0.225	0.950	0.089	0.011	1.000	Major in CEU & YRI	(39)
rs12913832	*HERC2*	Eye color	A	G	0.071	14	(13/0/1)	0.0002	0.325	0.792	0.00	0.00	0.00	Monomorphic in JPT & YRI, Major in CEU	(40)

SNP, single nucleotide polymorphism; A, major allele; B, minor allele; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; CEU, Centre European; HCB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo; YRI, Yoruban in Ibadan, Nigeria (http://www.hapmap.org/). HapMap: A haplotype map of genetic variations in different populations.

*HWE determined with the chi-square Hardy–Weinberg equilibrium test calculator (22), and *P*-values were calculated with the Michael H. Court's (2005–2008) online calculator Excel-based HWE test. *P* values <0.05 are not consistent with HWE. (https://www.researchgate.net/file.PostFileLoader.html?id=58dbf27a96b7e4fc194da329&assetKey=AS%3A477303651213312%401490809466020).

Korean, African American, Caucasian, and Pathan were analyzed with the in-house SNaPshot SBE multiplex system (see Fig. S1). The MAFs of all SNPs across these four populations were determined (Table S1).

DNA Typing of Bone Samples

To test the suitability of the multiplex system for challenging bone samples, 24 bone samples from 200- to 500-year-old mass graves were used for examination with the SNaPshot SBE multiplex. The DNA quantity of the analyzed bone sample ranged between 0 and 200 pg/μL as shown in Table S2. DNA was detectable in 17 from 24 samples. DNA amount of seven of those samples was below 10 pg/μL, while four samples ranged between 10 pg/μL and 100 pg/μL. The DNA yields of six samples were above 100 pg/μL. STR profiling applying the AmpFlSTR Identifiler PCR Amplification Kit with an elevated cycle number of 33 cycles resulted in nine full STR profiles and 11 partial profiles. Four of those samples did not show an interpretable profile (compare with Zar et al., 2013 (28)).

Generally, the interpretation of highly degraded low template (≤100 pg/μL) DNA profiles is very difficult (29). Therefore, according to the instructions of DNA interpretation rules (30), consensus profiles from SNP replicates were produced for each bone sample as shown in Table S2, similarly to the approach conducted by Cowen et al. (31). Among the 24 analyzed bone samples, 14 full SNP profiles (nine loci in each) and 10 partial SNP profiles (less than nine loci in each) were produced. Partial profiles were generated mainly from samples that showed DNA quantities below 5 pg/μL. All full SNP profiles were unique at least at one locus showing that no contaminations occurred during the analysis.

Besides, wild-type allele frequencies and mutant-type allele frequencies of all SNPs across bone samples were determined (Fig. 1). The minor allele frequencies (MAFs) of all SNPs [rs885479 (MC1R), rs26722 (SLC45A2), rs2031526 (DCT), rs7495174 (OCA2), rs4778241 (OCA2), rs4778138 (OCA2), rs1800414 (OCA2), rs1545397 (OCA2), and rs12913832 (HERC2)] across highly challenging bone samples were 0.11, 0.18, 0.25, 0.21, 0.25, 0.43, 0.07, 0.29, and 0.07, respectively (Table 3). To reveal the association of these samples with one population, the MAFs of each SNP of the bone samples were compared with minor allele frequencies of Pathan, European (CEPH Utah residents with ancestry from Northern and Western Europe—CEU), Chinese (Han Chinese in Beijing, China—HCB), Japanese (Japanese in Tokyo, Japan—JPT), and African (Yoruba in Ibadan, Nigeria—YRI) populations. MAFs of the populations were taken from the HapMap database (http://www.ncbi.nlm.nih.gov/SNP/) (32).

In our study, all SNPs were polymorphic across the bone samples. Two SNPs [rs26722 (SLC45A2), rs4778138 (OCA2)] met Hardy–Weinberg equilibrium (HWE) ($P < 0.05$) and seven SNPs [rs1800414 (OCA2), rs12913832 (HERC2), rs885479 (MC1R), rs2031526 (DCT), rs7495174 (OCA2), rs4778241 (OCA2), rs1545397 (OCA2)] were in disequilibrium as shown in Table 3. All in all, our results indicate that the skeletal remains are genetically closer to the Pathan population (Fig. 2). This suggests that this kit might be used for generating an allele frequency database to calculate the allele frequencies of genetic profiles of all populations. The main reasons for some allele frequencies to deviate from HWE might be environmental factors such as inbreeding, selection, and population substructure (33). Highly significant deviations from HWE show that the Hardy–Weinberg equation cannot exactly ascertain the allele frequencies of a population. In such cases, the allele frequency database may not be convenient to calculate the allele frequencies of genetic profiles in a population (34).

Conclusions

Common STRs and mini-STRs often fail to develop full DNA profiles from highly challenging DNA samples like bones. Due to DNA degradation and/or inhibition of the amplification, STR profiling is not successful and only partial profiles are generated. Adjusting PCR variables to optimize the reaction such as enhancing the number of PCR cycles increases the quantity of DNA products. However, the unpleasant side effect of this is the concomitant increase in stochastic effects, for example high stutter, allelic drop-in, allelic dropout, and allele peak height imbalance (35). Therefore, analysis of bone samples sometimes requires alternative approaches providing additional information for the identification of skeletal remains. In this study, SNP genotyping of bone samples was carried out with a novel in-house SNaPshot SBE multiplex system using nine SNPs. The results demonstrate that the multiplex system succeeded to yield profiles in most of the presented cases. The size of SNP amplicons is shorter than of conventional STRs and mini-STRs; therefore, the amplification of SNPs is more vigorous and stochastic effects occur less frequently (36,37). Even though the significance of this new multiplex system will be further verified using larger numbers of DNA samples in future experiments, this study presents a newly developed assay for SNP genotyping of highly challenging DNA samples that can provide crucial investigative leads related to phenotype- and ancestry-informative attributes in criminal investigations.

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Supporting Information

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

Figure S1. Examples of SNP profiles generated A) from blood sample of individual from Pathan population and B) from one bone sample.

Table S1. Major and minor allele frequencies of the 9 SNPs across Korean, African American, Caucasian and Pathan populations (20 samples/population).

Table S2. Quantification results of DNA from highly challenging old bone samples in pg/μL. DNA replicates were analyzed.