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ORIGINAL ARTICLE

Exploring of new Y-chromosome SNP loci using Pyrosequencing and the SNaPshot methods

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Abstract The single nucleotide polymorphisms on the Y chromosome (Y-SNP) have been considered to be important in forensic casework. However, Y-SNP loci were mostly population specific and lacked biallelic polymorphisms in the Asian population. In this study, we developed a strategy for seeking and genotyping new Y-SNP markers based on both Pyrosequencing and the SNaPshot methods. As results, 34 new biallelic markers were observed to be polymorphic in the Chinese Han population by estimation of allele frequencies of 103 candidate's Y-SNP loci in DNA pools using Pyrosequencing technology. Then, a multiplex system with 20 Y-SNP loci was genotyped using the SNaPshot™ multiplex kit. Twenty Y-SNP loci defined 56 different haplotypes, and the haplotype diversity was estimated to be 0.9539. Our result demonstrated that the strategy could be used as an efficient tool to search and genotype biallelic markers from a large amount of candidate loci. In addition, 20 Y-SNP loci constructed a multiplex system, which could provide supplementary information for forensic identification.

Keywords SNaPshot minisequencing · Pyrosequencing · Y-SNP · Forensic genetics

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Introduction

The single nucleotide polymorphisms on the Y chromosome (Y-SNP) have been considered to be important in forensic casework. Its simplicity is a great advantage when carrying out detective work on samples from the low quantity of DNA in small fragments and is conducive to be analysis with high throughput technologies [1, 2]. Furthermore, the regional affiliation emerging from the haplogroup of Y chromosome biallelic markers may be a useful factor for predicting the geographic origin of an unknown sample [3, 4]. In 2002, the Y Chromosome Consortium published a single parsimony tree showing the relationship among 153 haplogroups based on 243 binary markers [5]. In 2008, Karafet et al. [6] reported an extensively revised Y chromosome tree containing 311 distinct haplogroups and incorporating approximately 600 binary markers. Recently, the 1000 Genomes Project Consortium reported a map of human genome variation from whole-genome sequencing [7]. Compared to other chromosomes, however, the sequence variation on the Y chromosome has appeared to be relatively low [8] and exhibited extensive genetic differentiation, which resulted in markedly different haplogroup profiles distribution in different population. Espe-

cially, so far, there was still a lack of biallelic polymorphisms in the Asian population. Therefore, it is significant to develop effective techniques of looking for and genotyping more new Y-SNP markers, which can be applied in most forensic laboratories. In this study, we developed a strategy for seeking and genotyping new Y-SNP markers based on both Pyrosequencing and the SNaPshot methods.

Materials and methods

DNA samples and extraction

The samples were collected from 220 non-related male individuals and two non-related female individuals in Chinese Han population. DNA from EDTA blood was extracted using the salting-out method. DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Individual samples were diluted to 10 ng/μL using high-purity water. Each sample that was included in DNA pools for Pyrosequencing was quantified at least in duplicates and the DNA concentration adjusted if deviating from 10±0.5 ng/μL. To ensure accuracy of estimation of allele frequency, it was crucial that an equal amount of effective DNA template from each sample was included in the pool, as errors would skew the proportion of each genotype in DNA pool.

SNP selection from the pubic database

In order to search new Y-SNP loci suitable for forensic purposes in the Chinese Han population, we chose 103 Y-SNP loci from the public database dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), and they are shown in Table S1 (electronic supplementary material). The sequences of all Y-SNP markers were acquired from GenBank (http://www.ncbi.nlm.nih.gov), and allele frequencies were investigated in DNA pools using Pyrosequencing.

Pyrosequencing technique

Fifty-four individual DNA samples from 220 non-related male individuals were pooled (the size of DNA pool was adequate to find new polymorphic markers). The pooled DNA template for every SNP locus was amplified by PCR. The reaction consisted of 10× PCR buffer, 1 mol/L deoxyribonucleotide triphosphates (dNTPs), 25 mM $MgCl_2$, 1.5 U Taq polymerase (Tiangen), 10 pmol/μL 5′ biotin TEG-labeled, high-performance liquid chromatography purified primer, and 10 pmol/μL unlabeled primer (TaKaRa Biotechnology (Dalian) Co, Ltd.), 2 μL 10 ng/μL pooled DNA and sterile water to 50 μL final volume. Amplification reactions were performed in a GeneAmpPCR systems 9600 (Applied Biosystems, Foster City, CA, USA) using the following conditions: 94°C for 4 min; 50 cycles of [94°C for 30 s (58−67°C) for 30 s, 72°C for 45 s]; 72°C for 10 min; 4°C until removal from the thermocycler. Fifty cycles of amplification are recommended to deplete reagents and to facilitate similar amplification efficiency of samples [9].The biotin-ssDNA was then isolated and applied in the PSQ™ 96 system. Y-SNP loci analyses were performed using the PSQ™ 96 system in combination with the SNP reagent kit, according to the recommendations of the manufacturer (Pyrosequencing AB). Primers (concluding two PCR primers and one internal Pyrosequencing primer) were designed using PSQ™ Assay Design software. All frequencies in the samples were assessed by PSQ96 AQ software [9].

SNP selection for SNaPshot method

Firstly, according to the result of Pyrosequencing, we chose 12 Y-SNP loci markers that were polymorphisms and of value to establish haplotypes in studied Chinese population. In addition, based on the degree of polymorphism to be most informative in Chinese population, we chose eight Y-SNP loci in the phylogenetic tree [5, 6] by looking at the studies previously published [10–13]. Eight Y-SNP loci were respectively M9, M15, M88, M89 (rs2032652), M95, M122, M134, and M145. All of them were located in the basal branches of the phylogenetic tree and defined the major clades A–R [5, 6]. M145 defined the DE haplogroup, M89 defined the F haplogroup, M9 defined the K haplogroup, M15 defined the D1 haplogroup, M95 defined the O2a haplogroup, M122 defined the O3 haplogroup, and M134 and M88 respectively defined two haplogroup inside subclade O3a3c and O2a1. A total of 20 Y-SNP loci were combined in a multiplex and estimated using SNaPshot method.

SNaPshot technique

Design of PCR and extension primers

PCR and extension primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and Single Base Extension (SBE) Primer Version 1.1 software respectively [14]. The sequences of 20 Y-SNP loci PCR and extension primers are shown in Tables S2 and S3 (electronic supplementary material).

PCR conditions and purification of PCR products

Each primer pair was tested in singleplex PCR, respectively. Then, 20 Y-SNP loci were combined in one PCR multiplex

reaction. The multiplex reaction was carried out in a total volume of 25 μL using 12.5 μL of One Shot LA PCR Mix [TaKaRa Biotechnology (Dalian) Co, Ltd.], 10 ng DNA template, and 0.04–1.30 μM of PCR primers(see Table S2 for concentrations). Singleplex and multiplex amplification reactions were performed in a GeneAmpPCR systems 9600 (Applied Biosystems, Foster City, CA, USA) using the following conditions: 94°C for 5 min; 34 cycles of (94°C for 30 s, 60°C for 30 s, 72°C for 45 s); 72°C for 10 min; and 4°C until removal from the thermocycler. The negative controls (sterile water and DNA sample from two female samples) were performed for the multiplied PCR reaction. The products were analyzed by electrophoresis in 8% polyacrylamide gels.

To avoid participation of remaining dNTPs and primers in the subsequent primer-extension reaction, the PCR products were purified by adding 3 U shrimp alkaline phosphatase (SAP) [TaKaRa Biotechnology (Dalian) Co, Ltd.] and 5 U exonuclease I (EXO I) [TaKaRa Biotechnology (Dalian) Co, Ltd.] to 9 μL PCR product, and the mix was incubated at 37°C for 1 h. The enzymes were inactivated by incubation at 75°C for 15 min.

SBE reaction and purification of the SBE products

The single nucleotide primer extension reaction was carried out using the SNaPshot™ multiplex kit (Applied Biosystems). The single nucleotide primer extension reaction was performed in a volume of 10 μL containing 3 μL of PCR product, 0.02–0.50 μM of typing primers (see Table S3 for concentrations), and 3 μL of SNaPshot reaction mix (Applied Biosystems). The SBE reaction was performed using the following program: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. A positive control (provided with the kit) and negative controls (including female DNA, all reagents except DNA, as well as PCR products in the SNaPshot mix without the SNaPshot primers) were performed for the multiplied minisequencing reaction. The homogeneity of each primer was checked in singleplex minisequencing.

After the extension reaction, the unincorporated dideoxynucleoside triphosphates (ddNTPs) were removed by enzymatic treatment using 1 U of SAP to avoid comigration of [F] ddNTPs with the fragment of interest.

Capillary electrophoresis

Following post-extension treatment, 2 μL of cleaned minisequencing reaction product was then mixed with 7.5 μL Hi-Di formamide (Applied Biosystems) and 0.5 mL of GeneScan-LIZ 120 internal size standard (Applied Biosystems). After a denaturing step for 2 min at 95°C followed by cooling to 4°C, the products of single base extensions were separated on a 310 Genetic Analyzer (Applied Biosystems) following the manufacturer's recommendations. Then, samples were analyzed using GENEMAPPER ID V3.2 software (Applied Biosystems).

Detection of sensitivity and male–female mixed samples by SNaPshot technique

In order to evaluate the sensitivity of the assay, serial dilutions of DNA at concentrations of 1 ng/μL, 500, 300, 200, and 100 pg/μL DNA from two male samples were made, and different amounts of DNA were used in the 20-plex PCR and SNaPshot reactions. Amplification and minisequencing of mixed samples containing ratios of female and male DNA ranging between 1:1, 250:1, and 500:1 were also performed.

Result

Y-SNP selected by the Pyrosequencing technique

Our result showed that most of 103 candidate markers did not have polymorphisms; only 34 Y-SNP loci had higher allele frequencies in the studied pooled DNA sample (including 54 Chinese individuals) and allele frequencies ranged from 0.436/0.564 to 0.098/0.902. Allele frequencies of 103 candidate markers were provided as electronic supplementary material (Table S1, Fig. S1). Genotyping 54 individual DNA samples for 34 SNP loci by Pyrosequencing, we observed that nearly two thirds of 34 polymorphic markers linked with each other. The haplotypes that were constituted by 34 polymorphic markers were shown in Table S4 (electronic supplementary material). Eventually, we selected only 12 Y-SNP markers (rs17276358, rs17316592, rs16980426, rs9786707, rs16980711, rs9306845, rs17323322, rs11096433, rs13447354, rs17276345, rs9786479, and rs2075640) that were added to the further SNaPshot multiplex reaction.

Multiplex Y-SNP analysis by SNaPshot technique

Multiplex PCR

The length of PCR primers were selected between 20 and 27 base pairs long, and the sizes of amplicons ranged from 78 to 212 bp (Table S2), which facilitated analysis of samples that were highly degraded or carried low quantities of DNA. In a singleplex reaction, the estimated sizes were consistent with the expected sizes for all primer pairs. Then, in the multiplex assay with 20 amplicons, titrating PCR primer concentrations, which ranged from 0.04 to 1.30 μM,

obtained a balanced PCR multiplex system (Table S2). Finally, all tested samples were observed 20 bright multiplex bands with the correct amplicon sizes, and no band was in the negative control.

Multiplex minisequencing

The length of extension primer was adjusted at the 5′ end by addition of a poly-C tail or a poly (GACT) tail. The primer

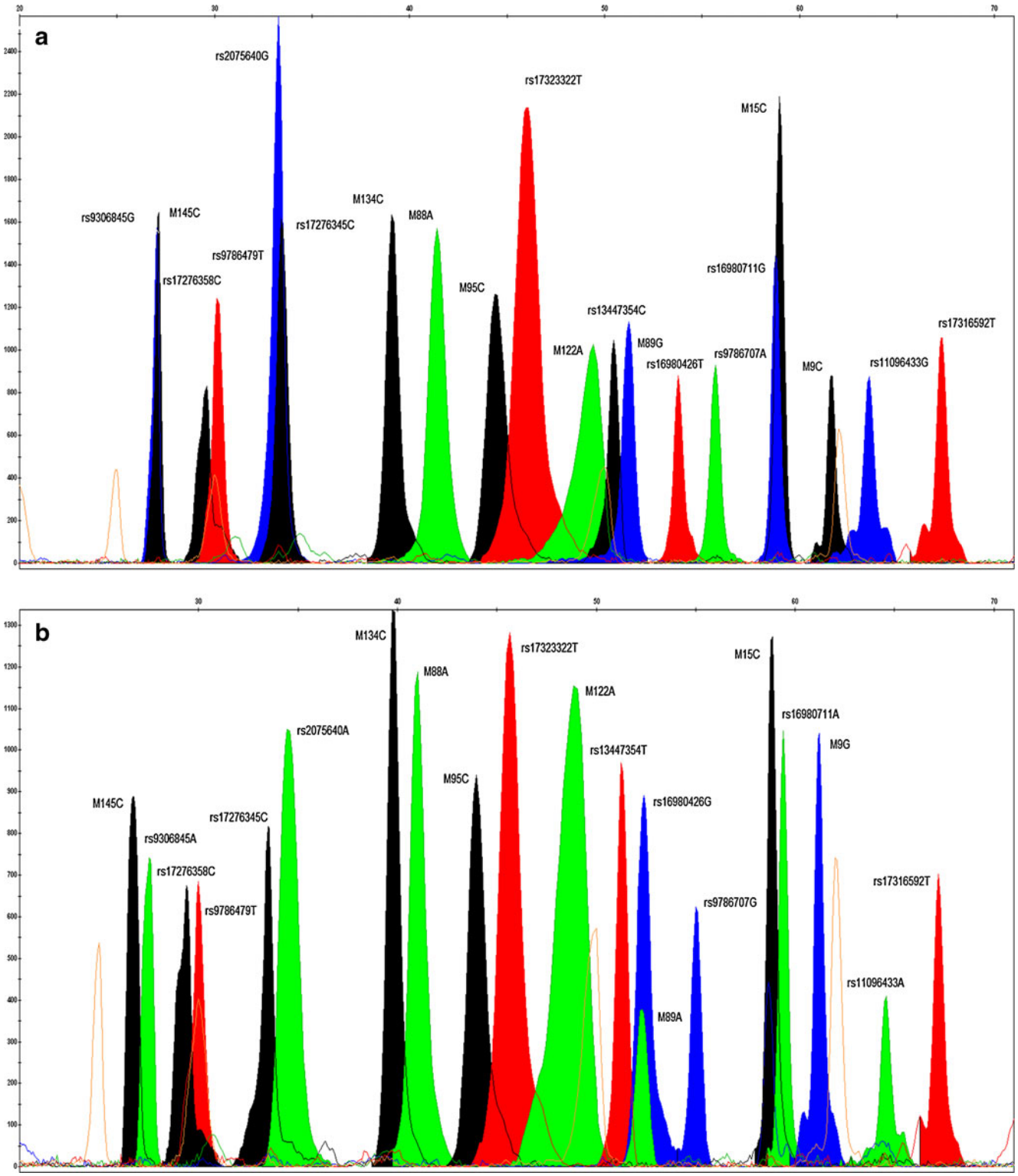

Fig. 1 **a**, **b** Result of Y-SNP typing by SNaPshot from different samples

sizes ranged from 21 to 68 nucleotides for 20 loci (Table S3). In the SNaPshot reaction, we increased or reduced the concentrations of some SNP primers to acquire balanced peak heights (Table S3). Finally, a full 20 Y-SNP profile with the balanced peak heights was obtained in each tested sample (Fig. 1). The negative control showed no peak in the Y-SNP loci range.

Sensitivity assays

The PCR reaction was observed to work efficiently with as little as 200 pg of DNA, and a complete SNP profile was obtained from only 500 pg of DNA in the SNaPshot reaction.

Male–female mixtures of DNA

In order to verify female DNA interference on the amplification of Y-SNP multiplexes, experiments with male–female mixtures were performed. The result demonstrated that female DNA did not influence the results of Y-SNP typing when added in concentrations more than 500 times the concentrations of male DNA. A full 20 Y-SNP loci profile in male–female mixtures sample was clearly shown in Fig. 2.

Y-SNP haplotypes frequencies

Our result showed all of 20 binary markers selected were polymorphisms in 220 studied Chinese male individuals. Fifty-six haplotypes with frequencies from 13.2% to 0.45% were identified (Table 1); the most frequent haplotype was type 1 (13.2%), followed by type 2 (11.4%), types 3–5 (each of them with a 7.7% frequency), and types 6 and 7 (6.8%). They were identified as the most abundant in the Chinese Han population. Haplotype diversity was estimated to be 0.9539.

Discussion

So far, there exists no database that could provide enough Y-SNP candidate markers in the Asian population. To constitute a multiplex system that was suitable for forensic application, we had to look for more new biallelic polymorphisms. Many technologies were now available for SNP detection [15, 16], among which estimation of SNP allele frequencies using the Pyrosequencing method in DNA pools has revealed its great advantages [9, 17–22]. Avoidance of genotyping individual samples for each SNP could markedly cut the number of reactions; the run and analysis times were also relatively short. In addition, previous studies have shown good agreement between pooled DNA and individual DNA measurements [17, 18], and a wide range of minor allele frequencies have been tested (5–50%) [18, 19]. However, some disadvantages of measuring SNP allele frequencies in DNA pools were that one was unable to construct haplotypes, and the maximum

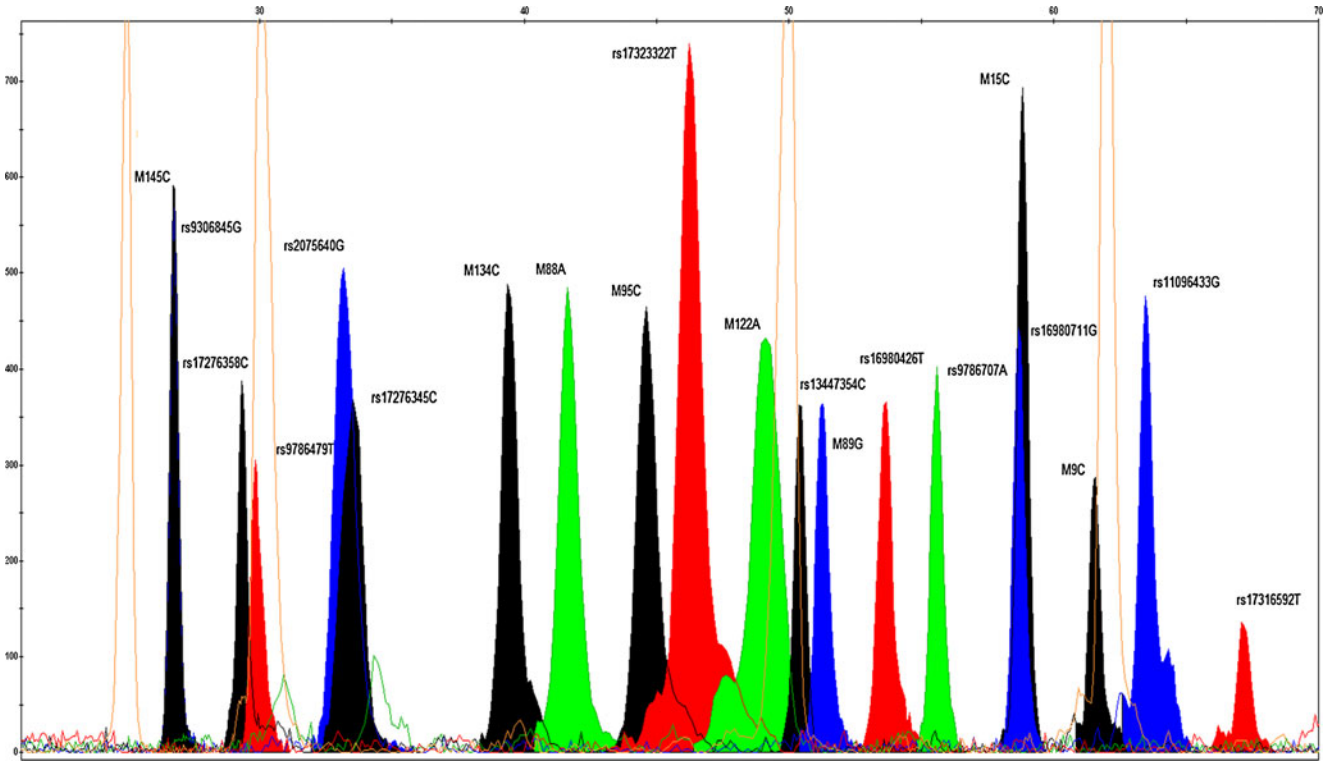

Fig. 2 Result of Y-SNP typing by SNaPshot of a mixture of male and female DNA 1:500

Table 1 Frequencies for 20 Y-chromosomal SNPs haplotypes in the Chinese Han population sample (n=220)

Type	SNP locus																				*F*
	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]	16[p]	17[q]	18[r]	19[s]	20[t]	
1	C	G	A	T	T	A	−	A	C	G	T	C	G	T	C	−	A	C	G	C	0.1318
2	T	G	A	T	G	A	+	A	C	G	T	T	A	T	C	−	A	C	T	C	0.1136
3	C	G	A	T	G	A	+	A	T	G	T	T	G	T	C	−	A	C	T	C	0.0773
4	C	G	A	T	G	A	+	A	C	G	T	T	G	T	C	−	A	C	T	C	0.0773
5	C	G	A	T	T	A	+	A	C	G	C	C	G	T	C	−	A	C	G	G	0.0773
6	C	G	A	T	T	A	+	A	C	G	T	C	G	T	C	−	A	C	G	C	0.0682
7	C	G	G	T	G	G	+	A	C	T	T	T	G	C	T	−	G	G	T	C	0.0682
8	C	A	G	G	G	G	+	A	C	T	T	T	G	C	T	+	G	G	T	C	0.0409
9	C	G	A	T	T	A	+	A	C	G	C	C	G	T	C	−	A	C	G	C	0.0318
10	C	G	A	T	G	A	+	G	T	G	T	T	G	T	C	−	A	C	T	C	0.0227
11	C	G	A	T	T	A	−	A	C	G	T	C	G	T	C	−	A	C	T	C	0.0182
12	C	G	A	T	T	A	−	A	T	G	T	C	G	T	C	−	A	C	G	C	0.0182
13	C	G	A	T	G	A	+	A	C	G	T	C	G	T	C	−	A	C	T	C	0.0136
14	C	G	A	T	G	A	+	A	C	T	T	T	G	T	C	−	A	C	T	C	0.0136
15	C	G	A	T	T	A	+	A	C	G	T	C	G	T	C	−	A	C	G	G	0.0136
16	C	G	G	T	G	G	−	A	C	T	T	T	G	C	T	−	G	G	T	C	0.0091
17	C	G	G	T	G	G	+	A	C	T	T	T	G	C	T	−	G	C	T	C	0.0091
18	C	A	G	G	G	G	+	A	C	T	T	T	G	C	T	−	G	G	T	C	0.0091
19	C	G	A	T	T	A	−	A	T	G	T	T	A	T	C	−	A	C	G	C	0.0091
20	T	G	A	T	G	A	+	A	T	G	T	T	A	T	C	−	A	C	T	C	0.0091
21	T	G	A	T	G	G	+	A	C	G	T	T	A	T	C	−	A	C	T	C	0.0091
22	C	A	A	G	G	G	+	A	C	T	T	T	G	C	T	+	G	G	T	C	0.0045
23	C	G	A	T	G	A	+	A	T	G	T	T	A	T	C	−	A	C	T	C	0.0045
24	C	G	A	T	T	A	+	A	C	G	C	C	G	T	C	−	A	C	T	G	0.0045
25	T	G	A	T	G	A	+	A	C	G	T	T	G	T	C	−	A	C	T	C	0.0045
26	C	G	A	T	T	A	−	A	C	G	T	C	G	T	C	+	A	C	G	C	0.0045
27	C	G	G	T	G	G	+	A	C	G	T	T	G	C	T	−	G	G	T	C	0.0045
28	C	G	A	T	T	A	−	A	C	G	C	C	G	T	C	−	A	C	G	G	0.0045
29	C	G	G	T	G	A	+	A	C	T	T	C	G	T	C	−	G	G	T	C	0.0045
30	C	A	G	G	T	G	+	A	C	T	T	T	G	C	T	+	G	G	T	C	0.0045
31	C	G	G	T	T	A	−	A	C	G	C	C	G	T	C	−	A	C	G	C	0.0045
32	C	G	A	T	T	A	−	A	C	T	T	T	G	C	T	−	A	C	G	C	0.0045
33	C	G	A	T	T	A	+	A	C	T	T	T	G	C	T	−	A	C	G	C	0.0045
34	C	G	A	T	T	A	−	A	C	G	T	C	G	T	T	−	A	C	G	C	0.0045
35	C	A	A	T	T	A	+	A	C	G	C	C	G	T	C	−	A	C	G	C	0.0045
36	C	A	A	T	T	A	+	A	C	G	C	C	G	T	C	−	A	C	G	G	0.0045
37	C	A	A	T	G	A	+	A	T	G	T	T	G	T	C	−	A	C	T	C	0.0045
38	C	G	A	T	G	A	+	A	T	G	T	T	G	T	T	−	A	C	T	C	0.0045
39	C	A	A	T	G	G	−	A	C	T	T	T	G	C	T	+	G	G	T	C	0.0045
40	C	G	A	T	G	A	+	A	C	T	T	T	G	T	C	−	A	G	T	C	0.0045
41	C	A	G	G	G	G	−	G	C	T	T	T	G	C	T	+	G	G	T	C	0.0045
42	C	G	G	T	T	G	+	A	C	G	T	T	G	C	T	−	G	G	T	C	0.0045
43	C	A	G	G	G	G	+	A	C	T	T	T	G	C	T	−	G	G	G	C	0.0045
44	C	G	A	T	T	A	−	A	C	G	C	C	G	T	C	−	A	C	G	C	0.0045
45	C	G	A	T	G	A	+	A	C	G	T	T	G	T	C	−	A	C	T	G	0.0045
46	C	G	A	T	G	A	+	A	C	G	C	C	G	T	C	−	A	C	G	C	0.0045
47	C	G	A	T	T	A	+	A	C	G	T	C	G	T	C	−	A	C	T	C	0.0045

Table 1 (continued)

Type	SNP locus																				*F*
	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]	16[p]	17[q]	18[r]	19[s]	20[t]	
48	C	G	A	T	T	A	−	A	C	G	T	C	G	T	C	−	A	C	G	G	0.0045
49	C	G	G	T	G	G	+	A	C	T	T	T	A	T	T	−	G	G	T	C	0.0045
50	C	G	A	G	T	A	+	A	C	G	C	C	G	C	C	−	A	C	G	G	0.0045
51	C	G	A	T	T	A	+	A	C	G	T	C	G	T	C	−	A	C	G	C	0.0045
52	C	G	A	T	T	A	+	A	C	G	T	T	G	T	C	−	A	C	T	C	0.0045
53	C	G	A	T	T	A	+	A	T	G	T	T	G	T	C	−	A	C	T	C	0.0045
54	C	G	A	T	G	A	+	A	C	G	C	C	G	T	C	−	A	C	G	G	0.0045
55	C	A	A	T	G	A	+	A	C	T	T	T	G	C	C	−	A	C	T	C	0.0045
56	C	G	G	G	G	G	+	A	C	T	T	T	G	C	T	−	G	G	T	C	0.0045

F frequencies

[a] rs11096433

[b] M145

[c] rs9306845

[d] rs9786479

[e] rs17276358

[f] rs2075640

[g] M134

[h] M88

[i] M95

[j] rs16980426

[k] rs17323322

[l] M122

[m] rs13447354

[n] M89

[o] rs9786707

[p] M15

[q] rs16980711

[r] M9

[s] rs17316592

[t] rs17276345

multiplexed level of pyrosequence-based typing was also limited [23, 24]. To circumvent from these drawbacks, we chose the SNaPshot method to genotype individual samples, which could analyze as many SNP loci as possible in a single reaction [25–27]. In addition, it was feasible when analyzing minimal DNA amounts or male–female mixtures of DNA and did not require high investments and new developments. Due to avoiding typing of single SNP and the potential high-throughput of the sequencer, the time and cost involved in this assay were reduced dramatically, so it has been widely applied in forensic laboratory.

Our result revealed that 103 candidate markers that have higher allele frequencies in other population showed lower polymorphisms in the studied Chinese population. In fact, only 34 biallelic markers were observed to be polymorphic. According to the further investigation, above all, we found that most of 34 biallelic markers linked with each other. They were completely meaningless for constructing haplotypes and increased more difficulty for selecting new Y-SNP loci. Table S4 (electronic supplementary material) showed the haplotypes that were constructed by 34 biallelic markers. We finally found out 12 SNP markers that were suitable to be added to the multiplex system. To enhance the discrimination power of the multiplex system in the Chinese population, we added eight biallelic polymorphisms that have been reported to be polymorphic in the Chinese Han population; most of them were East Asian specific and generally absent in other populations [10, 12, 13]. This kind of particular geographical distribution was useful to predict the possible geographic origin of any stain

contributor. The multiplex system of 20 Y-SNP loci that defined 56 haplotypes with relatively balanced haplotype frequencies (Table 1) could offer supplemental information for forensic identification works.

In the SNaPshot reaction, we checked the quality and size of extension primers in the simplex reaction; most loci got a single peak in the vicinity of expected sizes. However, due to the mobility of an oligonucleotide [11, 28], the estimated size of rs16980426G was similar as that of rs9786707G, although there were 3-bp discrepancies between the expected sizes of the two loci. We put the two loci in one reaction; some of the samples only gave one higher peak. In this case, we changed the length of rs16980426 extension primer from 53 to 51 bp. Despite this, there was a difference of only one nucleotide between the expected size of rs16980426 and M89; our result showed that their estimated sizes would not interfere with each other (Fig. 1).

Conclusions

Joint usage of the Pyrosequencing technique and the SNaPshot methods has shown great advantages in searching and genotyping biallelic makers. The former was preferable in selecting new significant markers from a larger number of candidate loci, and the latter could more effectively genotype individual sample and construct haplotypes. On the other hand, the multiplex system of 20 Y-SNP loci could be used as a powerful and simple tool for determining the origin of a male lineage and provided supplementary information for personal identification in the Chinese population.

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