Int J Legal Med (2012) 126:713–724
DOI 10.1007/s00414-012-0720-8

ORIGINAL ARTICLE

Y chromosome interstitial deletion induced Y-STR allele dropout in AMELY-negative individuals

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Received: 9 January 2012 / Accepted: 21 May 2012 / Published online: 6 June 2012

Abstract Short tandem repeat (STR) multiplexes with the amelogenin (AMEL) gene as a gender marker have been used as a routine tool of forensic DNA analysis. It has been reported that AMEL-based gender detection could misidentify a known male as a female due to the dropout of amelogenin Y (AMELY) allele. Other gender markers, such as Y-chromosomal short tandem repeat (Y-STR), may be a substitution of AMEL and help the sex determination. In current study, employing AmpF*l*STR® Sinofiler and AmpF*l*STR® Y-filer™ PCR Amplification kit, 18 AMELY-negative males were identified. Accordingly, the incidence of the AMELY dropout was 0.227‰ (18/79,304) in Chinese population. Sequencing of AMELY allele and analyzing of azoospermia factors region suggested that 3 out of 18 misidentifications were induced by mutations in the primer-binding region of the AMELY, while other 15 sex misidentifications were results of Y chromosome microdeletions with variant lengths. Moreover, variant combination patterns of AMELY dropout and Y-STRs deletions were also observed. Our data suggested that Y-STR locus dropout may indicate more problems, especially in the mixed sample's interpretation. Results of haplogroup prediction showed that seven AMELY dropouts combined with variant Y-STR deletions can be classified as the J2 subdivision, suggesting that some of these Y chromosomes might descend from a common ancestor.

Keywords Sex determination · Haplogroup · AmpF*l*STR® Y-filer™ PCR Amplification kit · Gender misidentification

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Introduction

Genotyping of amelogenin (AMEL) gene is widely used in commercially available short tandem repeat (STR) multiplex kits for the purpose of DNA-based sex detection. AMEL has two alleles which are amelogenin X (AMELX) and amelogenin Y (AMELY). The AMELX is located at Xp 22.1–22.3, and AMELY is located at Yp 11.2 [1]. PCR amplification of the AMELX and the AMELY, using the most common set of primers, produces two different fragments of 106 and 112 bp, respectively [2]. AMEL gene dropout, which mainly results from an amplification failure induced by deletion, translocation, and other mutations in the primer-binding site, could lead to sex misidentification [3, 4]. If the AMELY (112 bp) allele drops out, a male can be falsely genotyped as a female, and serious consequences arising therefrom will significantly affect case investigation and DNA database comparison.

Y-chromosomal short tandem repeats (Y-STRs) are powerful tool in current forensic DNA analysis and shown a particular utility in the male–female DNA mixtures and paternity identification. Y-STR marker system has been proved as an informative DNA marker system and been featured in a large database, Y-STR haplotype reference database (http://www.yhrd.org) [5], which allows rapid interrogation of population-specific frequencies of haplotypes and provides the likely

Table 1 Primer sequences designed for molecular diagnose of AZF region deletions

AZF region	STS	Sequence	Size (bp)
AZFa	SY84	SY84-F: 5′-(FAM) AGA AGG GTC TGA AAG CAG GT-3′ SY84-R: 5′-GCC TAC TAC CTG GAG GCT TC-3′	326
	SY86	SY86-F: 5′-(HEX) GTG ACA CAC AGA CTA TGC TTC-3′ SY86-R: 5′-ACA CAC AGA GGG ACA ACC CT-3′	318
AZFb	SY127	SY127-F: 5′-(HEX) GGC TCA CAA ACG AAA AGA AA-3′ SY127-R: 5′- CTG CAG GCA GTA ATA AGG GA-3′	272
	SY134	SY134-F: 5′-(FAM) GTC TGC CTC ACC ATA AAA CG-3′ SY134-R: 5′-ACC ACT GCC AAA ACT TTC AA-3′	303
AZFc	SY254	SY254-F: 5′-(HEX) GGG TGT TAC CAG AAG GCA AA-3′ SY254-R: 5′-GAA CCG TAT CTA CCA AAG CAG C-3′	381
	SY255	SY255-F: 5′-(HEX) GTT ACA GGA TTC GGC GTG AT-3′ SY255-R: 5′-CTC GTC ATG TGC AGC CAC-3′	124
Control	SRY	SRY-F: 5′-(FAM) GAA TAT TCC CGC TCT CCG GA-3′ SRY-R: 5′-GCT GGT GCT CCA TTC TTG AG-3′	465
	ZFX/Y	ZFX/ZFY-F: 5′-ATT GCG ACC ACA AGA GTT-3′ ZFX/ZFY-R: 5′-(FAM) GAA TAT GGC GAC TTA GAA CA-3′	219

population group or geographic location of samples [6]. It has been reported that some missed Y-STRs in Y-filer™ or PowerPlex® Y profile were associated with AMELY dropout [7–11]. These Y-STRs null loci may be the consequence of Y chromosome interstitial deletions.

In this study, 18 AMELY negatives were observed out of 79,304 males from Chinese population. Employing the AmpF*l*STR® Y-filer™ kit containing 16 Y-STRs loci, 14 specific haplotypes of AMELY negatives were observed. The microdeletion types of the short and the long arm of Y chromosome were analyzed, and the possible mechanisms of these deletions were discussed.

Materials and methods

DNA samples

Unrelated male blood samples (79,304) were collected with Whatman™ FTA® cards (GE Healthcare, Piscataway, NJ). A 1.2 mm disc was punched out by BSD puncher (BSD Robotic,

Table 2 Summary of frequencies of AMELY-negative male in global population groups

Country	Population	No. of nulls/individuals studied	Frequency (%)	Reference
Sri Lanka	Sri Lanka	2/24	8.333	[25]
India	Indian	5/270	1.852	[26]
India (whole)	Indian (caste and tribes)	10/4,257	0.235	[27]
Nepal	Nepalese	5/77	6.494	[24]
	Nepalese	9/769	1.170	[28]
Austria	Austrian	5/28,182	0.018	[15]
Italy	Italian	1/13,000	0.008	[17]
Spain	Spanish	1/768	0.130	[29]
Israel	Israelite	1/96	1.042	[30]
Australia	Mixed	22/109,000	0.020	[16]
Malaysia	Indian	10/315	3.175	[9]
	Malay	2/334	0.599	[9]
	Chinese	0/331	0	[9]
Singapore	Indian	3/175	1.714	[9]
	Malay	1/182	0.549	[9]
	Chinese	0/210	0	[9]
China	Chinese	3/8,087	0.037	[14]
	Chinese	18/79,304	0.023	Current study

Table 3 Y-STR haplotypes of the 18 AMELY negative males typed with Y-filer™

Sample	Haplogroup	DYS456 (13–18)	DYS389I (10–15)	DYS390 (18–27)	DYS389II (24–34)	DYS458 (14–20)	DYS19 (10–19)	DYS385 a/b (7–25)	DYS393 (8–16)	DYS391 (7–13)	DYS439 (8–15)	DYS635 (20–26)	DYS392 (7–18)	Y-GATA_H4 (8–13)	DYS437 (13–17)	DYS438 (8–13)	DYS448 (17–24)
S8	E3a	–	14	23	29	–	16	11/19	14	10	14	21	11	11	14	10	21
S11	E3b	13	12	24	28	20	16	14/18	14	10	12	23	13	12	14	10	20
S15	I1b1b	15	12	23	28	–	15	12/15	12	10	12	21	12	12	15	11	19
S5	J1	15	12	23	28	16	15	12/21	12	10	11	22	13	12	14	10	21
S17	J2a1k	14	13	23	29	–	14	14/17	12	10	12	21	11	12	15	9	21
S2	J2b	13	12	25	28	–	15	13/17	12	10	14	22	11	–	15	9	19
S3	J2b	13	12	25	28	–	15	13/17	12	10	14	22	11	–	15	9	19
S6	J2b	13	12	24	28	–	15	14/16	12	11	12	21	11	11	15	9	18
S7	J2b	13	12	24	28	–	16	14/16	12	11	12	21	11	11	15	9	18
S10	J2b	13	13	24	29	–	15	14/18	12	10	12	21	11	10	15	9	18
S16	J2b	13	12	23	28	–	17	13/17	12	10	12	21	11	11	15	9	18
S9	K2	17	13	23	29	16	15	13	13	10	12	19	14	12	14	10	18
S13	L	15	–	23	–	20	15	12/16	12	11	–	19	12	12	–	10	19
S14	L	15	12	24	28	–	14	13/14	12	11	12	20	14	12	15	11	20
S12	N	16	13	21	29	–	14	11/14	13	10	11	21	15	12	14	10	19
S4	Q	15	12	23	29	–	–	12	12	10	13	23	12	12	15	11	19
S1	R1a	15	–	–	–	15	15	–	13	–	–	–	–	–	–	–	–
S18	R1b	15	–	–	–	–	–	–	12	–	–	–	–	–	–	–	–

S13 and S17 were from casework samples, while the others were all from DNA database. Number within brackets below the loci names indicated the various alleles present in the Y-filer™ allelic ladder. Haplogroups were estimated with Athey's haplogroup predictor software (http://www.hprg.com/hapest5/hapest5a/hapest5.htm). – No alleles present (null)

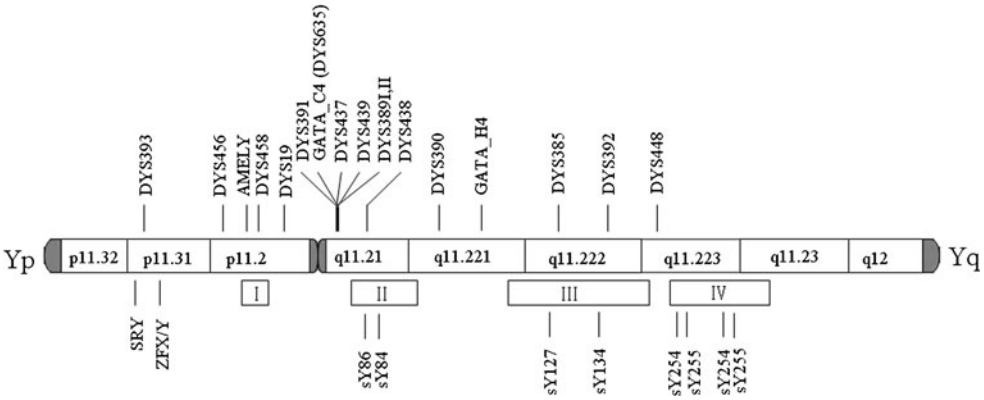

Fig. 1 Schematic of human Y chromosome. This schematic shows approximate locations of the 16 Y-STR loci from Y-filer™, AMELY, and eight STSs in AZF regions. Region I contains the AMELY-DYS458 deletion; regions II, III, and IV are the AZF-related regions

Queensland, Australia) and purified following the standard protocol. DNA from casework sample was extracted by TECAN Freedom EVO 100-4 automatic workstation (TECAN, Mannedorf, Switzerland), following the magnetic bead protocol (EQ1000 forensic DNA extraction kit).

Genotyping of autosomal STRs and Y-STRs

Amplifications of FTA samples were performed directly on the purified discs, following the demonstration of the manufacturer on a GeneAmp® PCR System 9700 (Life Technologies, Carlsbad, CA). For magnetic bead extracted DNA, approximately 1–1.2 ng DNA per assay was used. Autosomal STRs and Y-STRs were amplified, respectively, by using the AmpF*l*STR® Sinofiler PCR Amplification kit (Life Technologies) and the AmpF*l*STR® Y-Filer™ PCR Amplification kit (Life Technologies). PCR products were separated and detected on an ABI 3130*xl* Genetic Analyzer (Life Technologies), following manufacturer's recommendations. Electrophoretic results were analyzed using GeneMapper® ID software v3.2 (Life Technologies).

Table 4 Chromosomal localization of Y-STRs

Markers	Chromosomal localization	
	Arm	Position (Mb)
DYS393	Yp	3.17
DYS456	Yp	4.31
AMELY	Yp	6.73
DYS458	Yp	7.91
DYS19	Yp	10.11
DYS391	Yq	12.61
GATA_C4 (DYS635)	Yq	12.81
DYS437	Yq	12.90
DYS439	Yq	12.95
DYS389I	Yq	13.05
DYS389II	Yq	13.05
DYS438	Yq	13.37
DYS390	Yq	15.71
GATA_H4	Yq	17.18
DYS385a	Yq	19.18
DYS385b	Yq	19.20
DYS392	Yq	20.97
DYS448	Yq	22.70

Detection of Y chromosome deletions

Microdeletion of Y chromosome is a major cause of Y-STR locus dropout. Deletion in the azoospermia factor (AZF) region is associated with inability to produce sperm and has become a reliable tool of molecular diagnose of the Y chromosome microdeletion [12]. In current study, to understand the pattern of Y-STRs dropout occurred in our samples, we designed a multiplex PCR reaction according to three major AZF regions: AZFa, AZFb, and AZFc [13] (Table 1). A 25 ul total volume PCR reaction contains: 10 ul AmpF*l*STR® PCR Reaction Mix, 5 ul mixture of eight pairs of primers (sY84, sY134, SRY, ZFX/Y, sY86, sY127, sY254, and sY255 equals to 0.8, 0.8, 0.8, 0.8, 1.1, 0.4, 0.15, 0.15, respectively), 5 ul template DNA (approximately 1 ug), 0.5 ul AmpliTaq Gold® DNA polymerase, and 5 ul water. Amplification started with an initial denaturation at 94 °C for 10 min, followed by 28 cycles consisting of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (60 s at 65 °C), and ended with an elongation step of 60 min at 65 °C and cooling to 4 °C. Reaction products were denatured at 95 °C for 3 min, chilled quickly on ice for 3 min, and then separated on an ABI 3130*xl* Genetic Analyzer. The fluorescent fragments were analyzed using the GeneMapper® ID software v3.2.

Y chromosome haplogroup prediction

Based on Y-STR haplotypes, Y chromosome haplogroup was predicted using the Y haplogroup predictor program (http://www.hprg.com/hapest5/hapest5a/hapest5.htm).

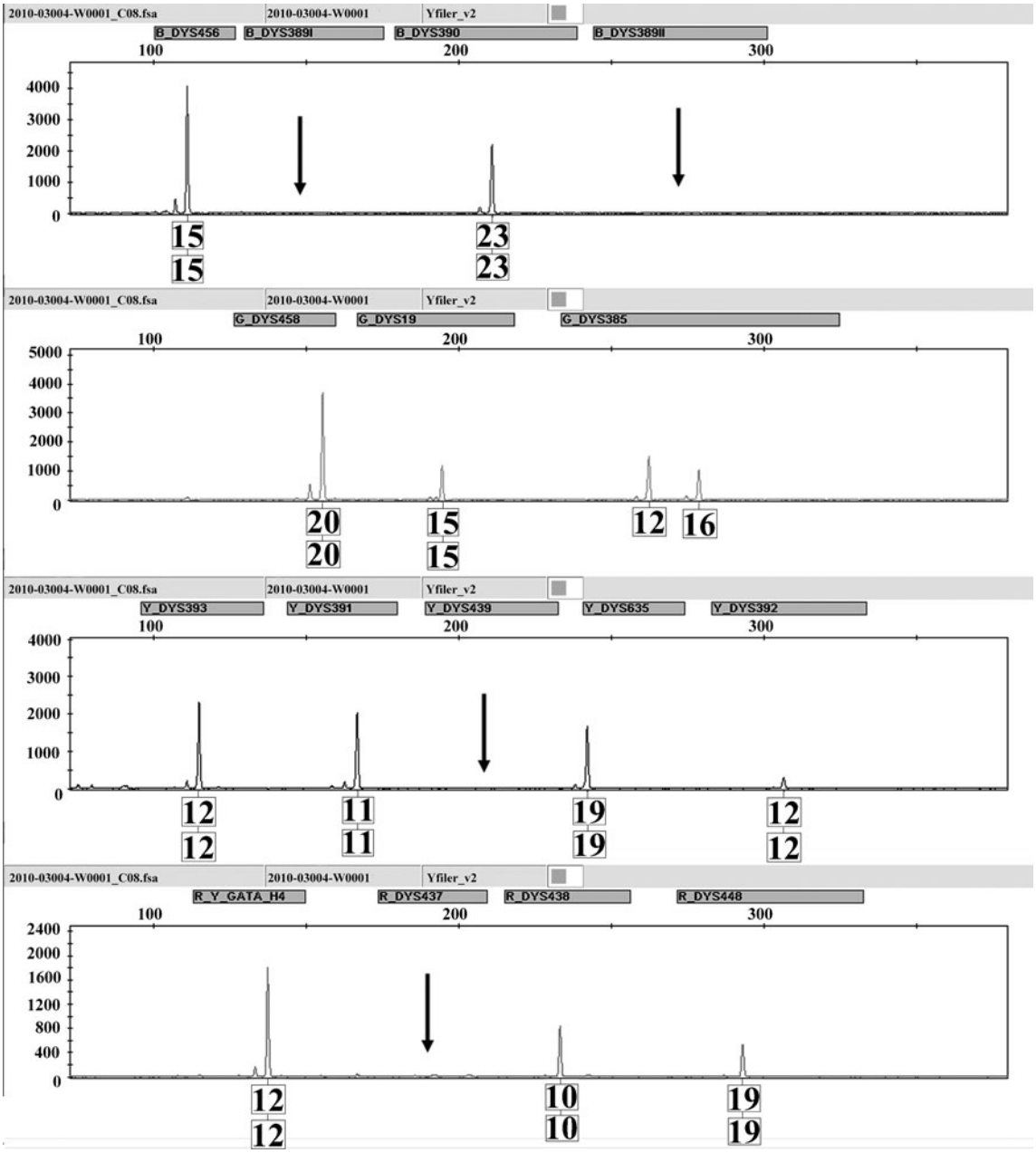

Fig. 2 Y-STRs haplotype of S13. In sample S13, AMELY allele, DYS437, DYS439, DYS389I, and DYS389II were absent simultaneously

Results and discussion

AMELY null frequencies

The AMEL gene integrated in most commercial STR multiplex kits is routinely used as a gender determination locus of the DNA samples that collected for the purpose of criminal investigations and DNA database. In current study, we identified 18 gender misidentifications by the joint use of commercial AmpF*l*STR® Y-Filer™ kit. All these 18 samples (S1–S18) showed a single homozygotic peak at 106 bp (AMELX). Accordingly, the frequency of AMELY null allele was 0.227‰ (18/79,304) in Chinese population in the North of China, which was similar with that in a previous study (0.37‰, $p>0.05$) based on 8,087 Chinese male samples from the South of China [14]. This value was also close to the data from Austria (5/28,182, 0.18‰) [15] and Australia (22/109,000, 0.2‰) [16], but different with some other populations (Table 2). A reasonable explanation of the differences among global populations is the amount of samples used in the study. Y-STR haplotypes of 18 AMELY negative individuals in current study were observed and sorted by the haplogroup (Table 3).

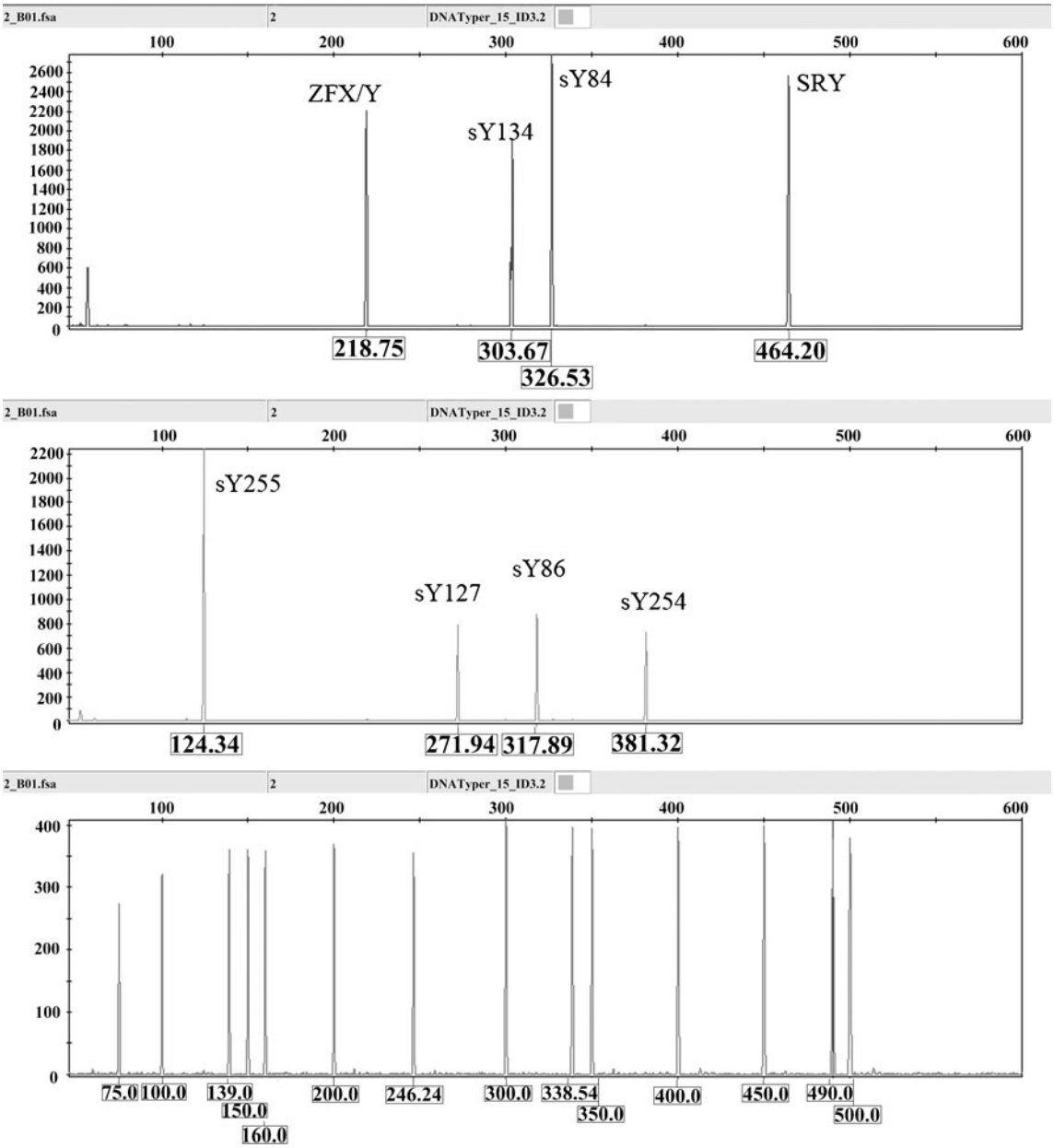

Fig. 3 Molecular diagnosis result of AZF regions in S2–S12 and S14–S17. All these 15 samples have complete profiles consist of eight STSs

Complete Y-STR profile

As showed in Table 3, samples S5, S9, and S11 were detected as AMELY allele-negative samples, but they still showed a complete Y-STR profile of the AmpF*l*STR® Y-Filer™ PCR Amplification kit. Sequencing results suggested that these three sex misidentifications resulted from point mutations occurred in the primer-binding region of the AMELY allele (data not shown). Since different kit might use different AMEL primer, this sort of sex misidentification may not appear in other PCR kits.

Limited Yp11.2 deletion (AMELY-DYS458)

In 18 AMELY-negative males, S6, S7, S10, S12, S14, S15, S16, and S17 only showed an absence of the DYS458 locus (Table 3), indicating that a microdeletion covering the DYS458 locus may happen at the short arm of Y chromosome. As showed in Fig. 1, AMELY and DYS458 locus are in the same region of Yp11.2 and very close to each other. Such small physical distance between the two loci may be an explanation of the high-frequency AMELY-DYS458 codeletion (8/18, 44 %), which was observed in Chinese population.

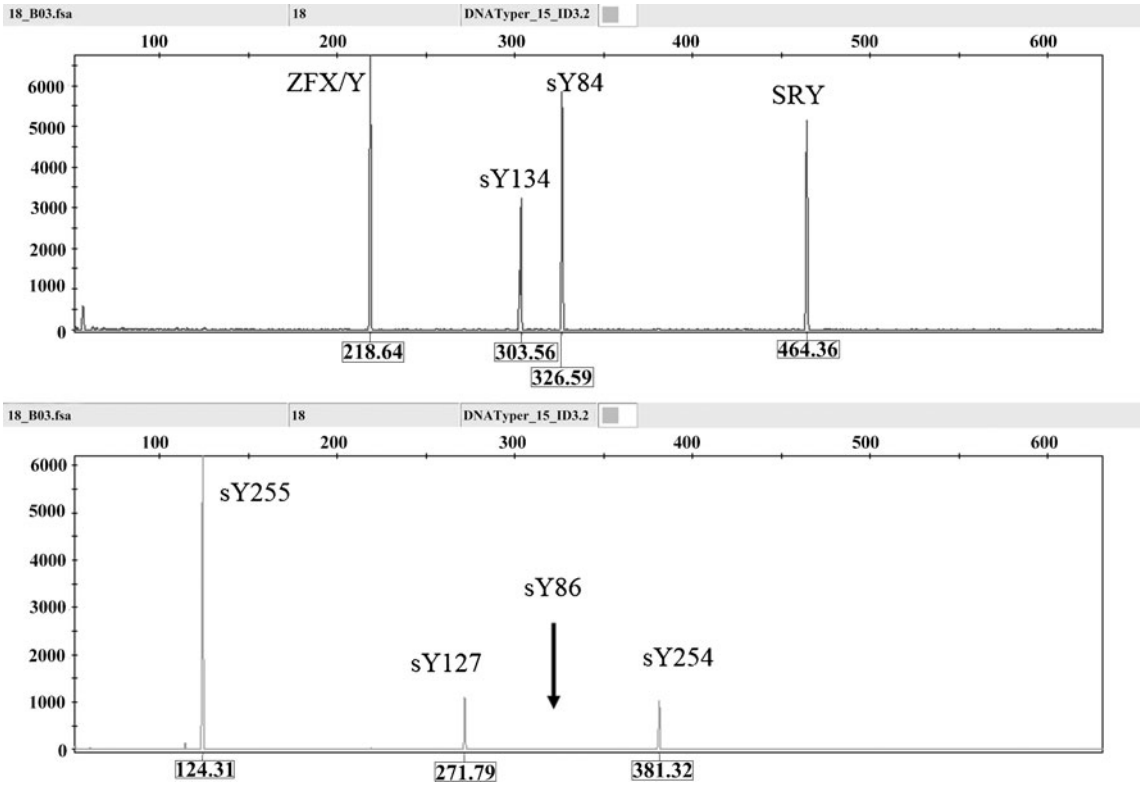

Fig. 4 Molecular diagnosis result of AZF regions in S13. STS sY86 was absent in S13

Thus, the DYS458 null might serve as a stronger indication of the AMELY negative. It has been reported that AMELY-DYS458 deletion region was located at the pericentromeric region of the Y chromosome short arm and been confirmed that the extent of this deletion spanned approximately 2.5 Mb [7, 8, 10, 17].

Large Yp11.2 deletion (DYS456-AMELY-DYS458/ AMELY-DYS458-DYS19)

DYS456 is located 2.42 Mb from the AMELY, and DYS19 is away from DYS458 at approximately 2.2 Mb (Table 4). In 18 AMELY dropout samples, S4 presented AMELY-DYS458-DYS19 deletion, and S8 displayed DYS456-AMELY-DYS458 deletion (Table 3). These two deletion patterns suggested that a larger deletion region existed in the Yp11.2 region, which covered loci DYS456, AMELY, DYS458, and/or DYS19 (Fig. 1). Similar result was reported by Ou et al. [14]. They showed a same deletion sized at least 3.6 Mb in the Yp11.2 region (DYS456-AMELY-DYS458) in Chinese population of the South China.

AMELY-DYS458-GATA_H4 deletion

In 18 AMELY-negative males, S2 and S3 showed absences of AMELY, DYS458, and Y-GATA_H4 locus. As showed in Fig. 1, Y-GATA_H4 is located at Yq11.221 and is close to AZFb region, which is one of the three major "hotspots" on the long arm of the Y chromosome related with azoospermia. Chang et al. [7] also reported the same deletion in India. It is hard to explain why two deletions happened simultaneously in such far apart regions (10.45 Mb from AMELY to Y-GATA_H4). Possible explanation is that two random deletion events (AMELY-DYS458 and GATA_H4) occurred simultaneously or a single large deletion covered these three loci, but the presence of an additional deletion at Y-GATA_H4 in Chinese males may be worth further investigation.

AMELY-DYS437-DYS439-DYS389I-DYS389II deletion

Twelve Y-STRs are located on the long arm of Y chromosome (Table 4). In S13 which was an evidence sample of a rape case we observed, allele absences of DYS437, DYS439, DYS389I, and DYS389II were detected (Fig. 2). All these four loci were located within the AZFa segment (Fig. 1), and suggested that such deletion might be a consequence of the AZFa region deletion [18]. Interestingly, locus DYS438, which was also located in AZFa region, still presented in this sample (Fig. 2), indicating an incomplete deletion of AZFa region. Compared with the standard molecular analysis pattern (Fig. 3), S13 presented STS marker sY84 and lost the marker sY86 in the AZFa region (Fig. 4). An explanation is that the missed loci (DYS437, DYS439,

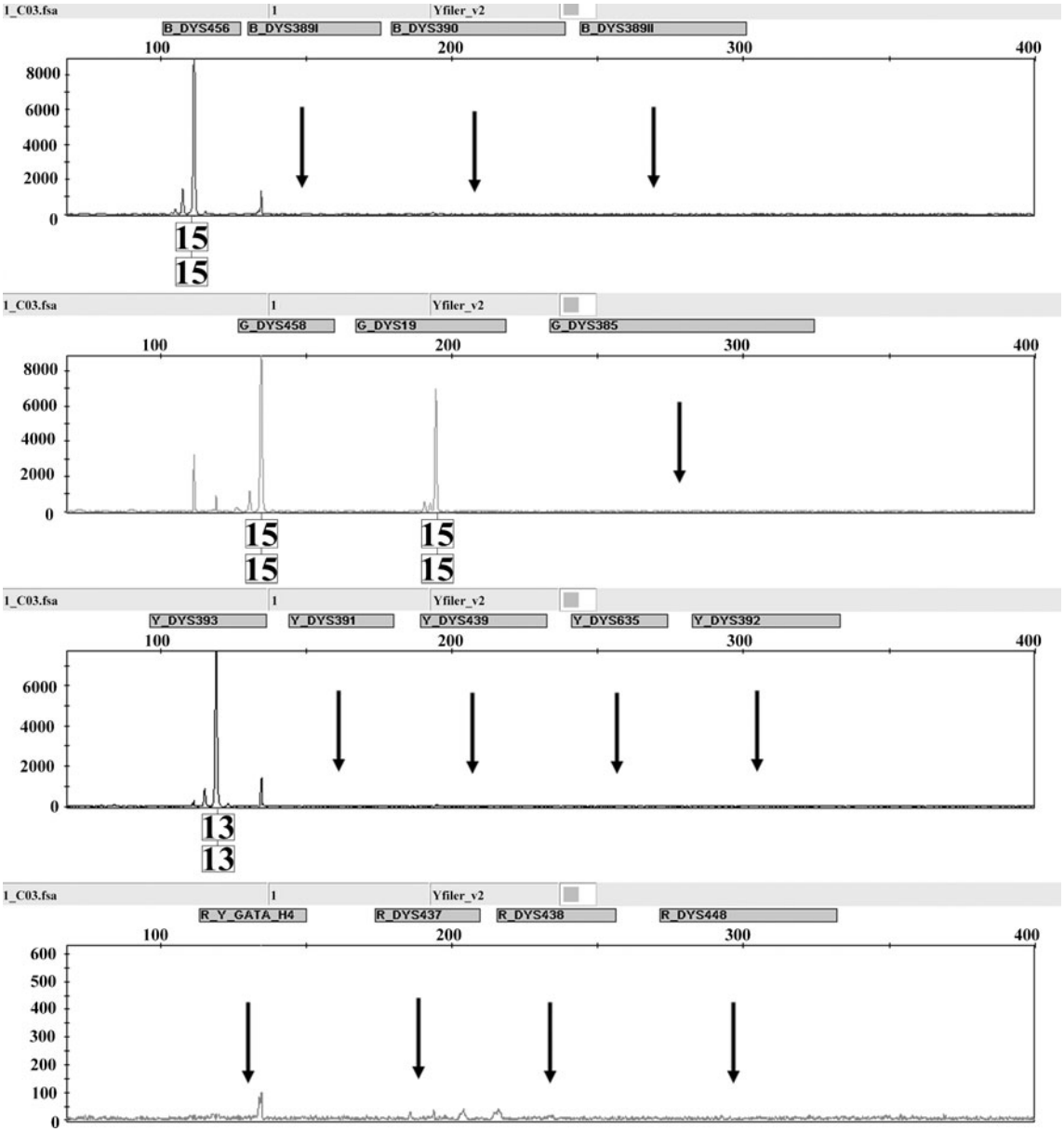

Fig. 5 Y-STR haplotype of S1. Negative amplifications were observed on the most loci of the Y-filer™ multiplex kit except for DYS456, DYS458, DYS19, and DYS393

DYS389I, and DYS389II) were close to sY86, and the presented locus DYS438 was close to sY84. Mechanism of such selective deletion has not been elucidated.

Long arm loss of Y chromosome and partial XY chromosome translocation in an XX man

S1 was identified as an XX man, which is characterized by an erroneous exchange happened in the nonrecombining regions of X and Y chromosome. Such chromatin exchange could result in a transfer of the SRY gene to the X chromosome [19]. Figure 5 showed that S1 carried two distal Yp Y-STRs, DYS393, and DYS456 (which have been confirmed to be translocated onto the short arm of one of his X chromosome, data not shown) and other two Yp Y-STRs, DYS458, and DYS19 (which were proximal to the absent AMELY). AMELY and all Y chromosome long arm markers were undetectable (Fig. 1). Molecular detection of AZF region confirmed the loss of Y chromosome long arm, since only ZFX/Y and SRY (located at on Y chromosome short arm) can be detected (Fig. 6). The most likely explanation of this discrepant pattern is that a paracentric inversion, transferring DYS19 to the distal IR3 element, is followed by translocation of a terminal segment of Y chromosomal material including SRY, DYS393, DYS456, DYS19, and DYS458 onto the X chromosome (Fig. 7) [6, 20]. The

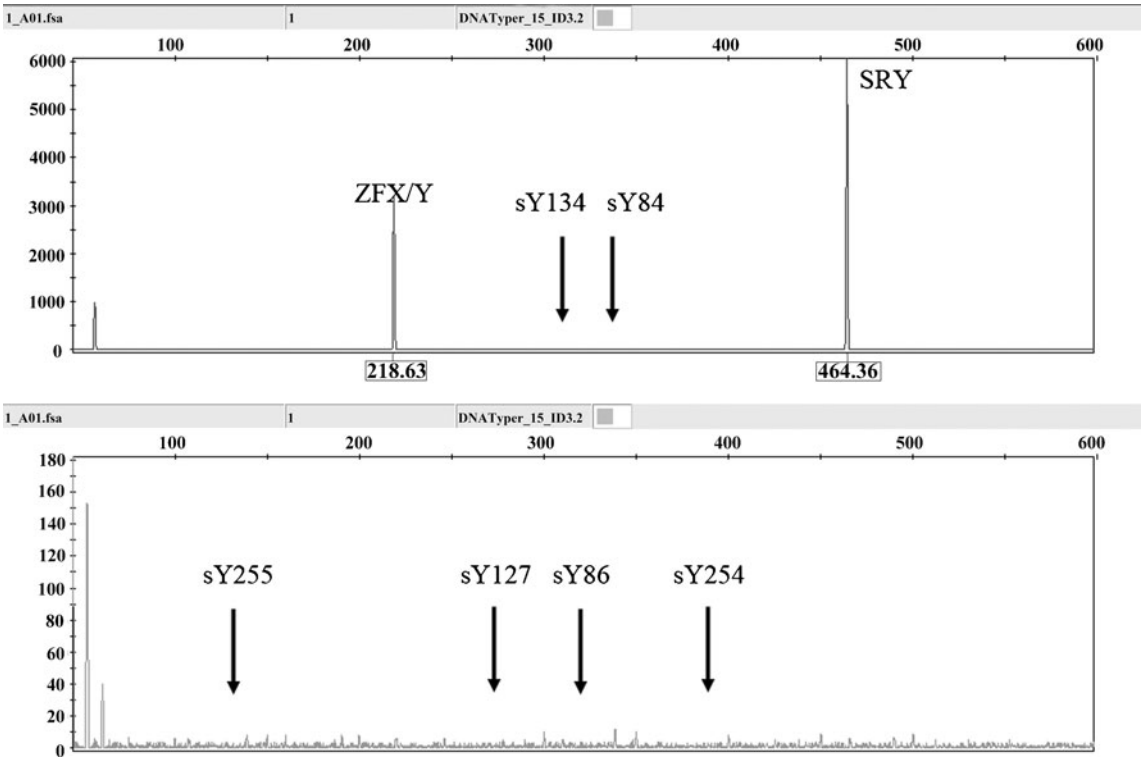

Fig. 6 Molecular diagnosis result of AZF regions in S1 and S18. Only STS ZFX/Y and SRY present in S1 and S18

partial short arm close to AMELY locus and the entire Y chromosome long arm are missing. Ou et al. [14] reported a similar deletion in Chinese population in the South of China.

Loss of most of Y chromosome in an XX man

S18 was another XX man and showed a different Y-STRs pattern compared with S1. Only DYS456 and DYS393 loci were detected in S18 (Fig. 8), suggesting a deletion spanning the major part of Y chromosome from Yp11.2 up to the whole long arm. This origin differed from S1, which IR3 elements did not transpose DYS19 into the distal IR3 region. AMELY together with DYS458, DYS19, and the whole long arm of Y chromosome were deletion, while the distal Yp markers, including SRY, DYS393, and DYS456, were translocated onto the short arm of X chromosome. Molecular detecting result of the deletion on Y chromosome long arm was the same as S1 (Fig. 6).

Y-STR nulls have effect on the interpretation of mixture

Mixtures of body fluids from two or more male individuals are frequent in forensic cases. It is possible that Y-STR nulls might effect the interpretation of this type of samples. For example, in a two males' mixture, if the mixed profile shows two alleles at various loci as well as the DYS458 locus

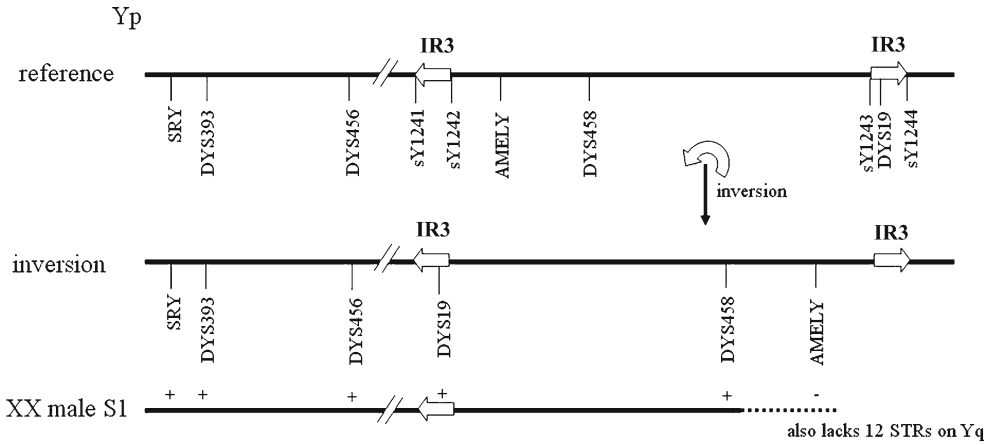

Fig. 7 Schematic of putative mechanism for the inversion and deletion occurred in XX male S1 (modified after Balaresque et al. [6]). DYS19 was transfer to the distal IR3 element by a paracentric inversion. Then, the terminal segment of Y chromosome was translocated onto the X chromosome

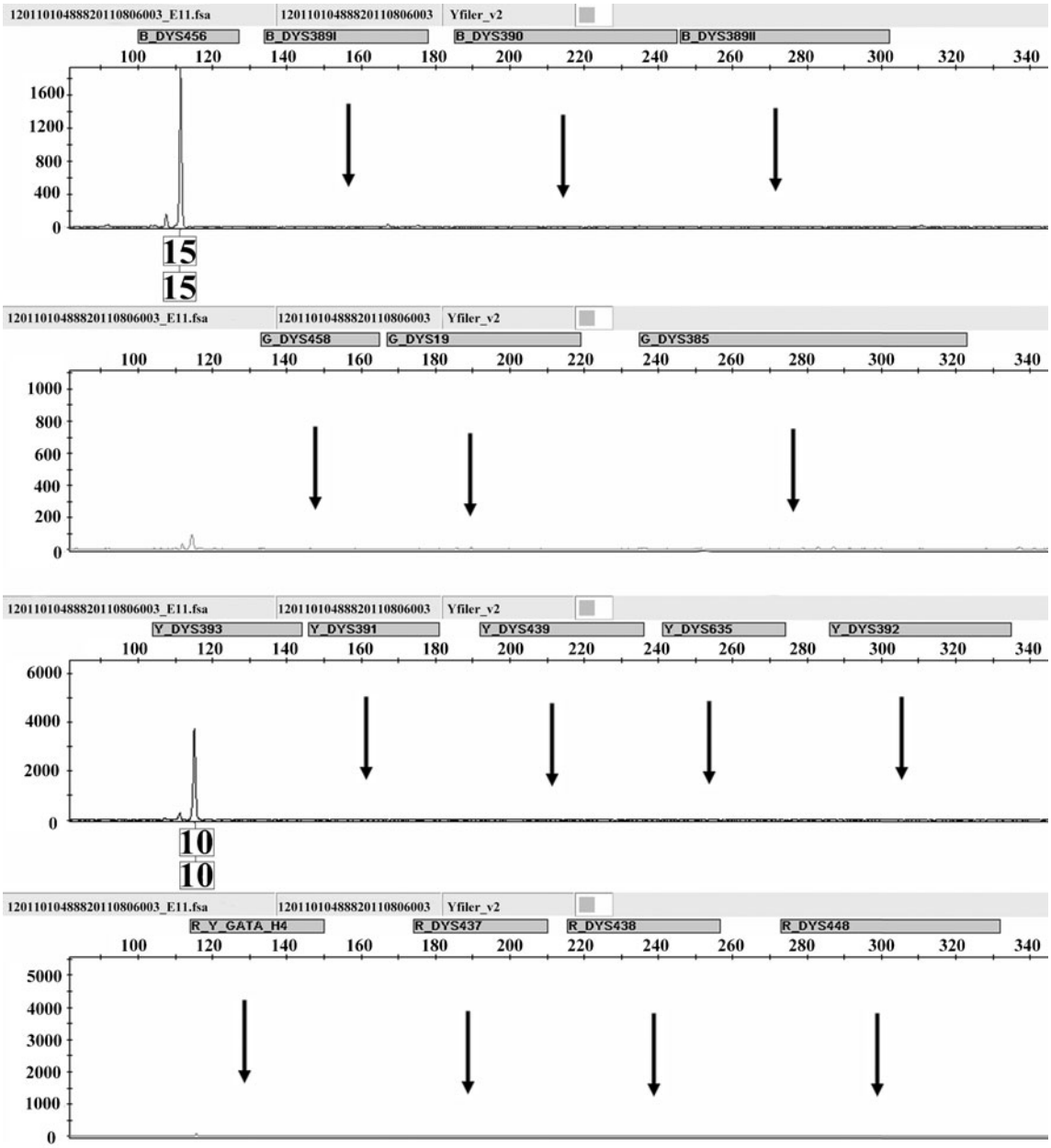

Fig. 8 Y-STR haplotype of S18. Sample S18 amplified successfully only on DYS456 and DYS393

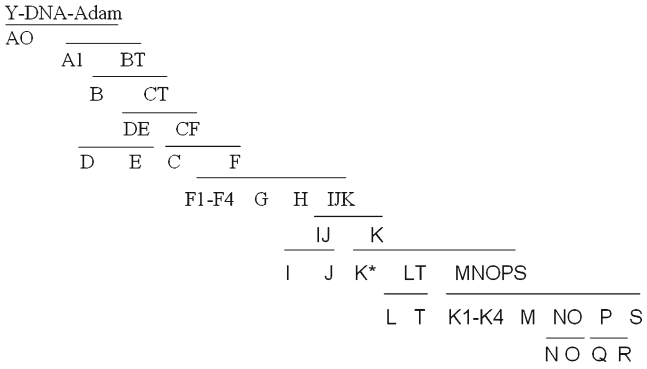

Fig. 9 Evolutionary tree of human Y chromosome DNA haplogroups

displays only an allele 16, one possible explanation is that both males have the same allele 16, and another explanation is that one male has an allele 16, and the other has a null allele. Obviously, the likely suspect, which has a null allele at the DYS458 locus, might be falsely excluded if this deletion was not taken into account. Generally, the number of individuals involved in male-mixed stains may be easier to decipher with the Y-STRs than with more complicated autosomal STRs. However, it will be difficult to determinate the number of contributors due to the absence of some Y-STR alleles as a consequence of the Y chromosome fragment deletion.

Haplogroup of the AMELY-negative males

Human Y chromosome DNA haplogroup is a haplogroup defined by differences in the nonrecombining portions of the Y chromosome. Typically, Y-SNPs are described as defining haplogroups and can be useful in DNA ancestry studies. The Y chromosome consortium has established a system of defining Y-DNA haplogroups by letters A through to T, with further subdivisions using numbers and lower case letters [21]. Y chromosomal Adam is the name given by researchers to a theoretical male who is the most recent common patrilineal (male lineage) ancestor of all living humans [22]. The evolutionary tree of human Y chromosome DNA haplogroups was summarized in Fig. 9.

Due to the limited amount of samples, it is difficult to perform supplementary test that would help to define accurately the haplogroup membership (e.g., Y-SNP typing). Thus, we chose the Y haplogroup predictor program and employing Y-STRs haplotype to make the Y chromosome haplogroup prediction. Result showed seven individuals (S2, S3, S6, S7, S10, S16, and S17) fallen into haplogroup J2 (defined by mutation M172 genetic marker), and six of them (S2, S3, S6, S7, S10, and S16) belonged to the subclade J2b (defined by M12 genetic marker), as well as S17 belonged to the subclade J2a (defined by M410 genetic marker). The Y chromosome J2 lineage has been estimated as 18,500±3,500 years ago and was mainly found in the Fertile Crescent, the Caucasus, Anatolia, the Balkans, Italy, the Mediterranean littoral, and the Iranian plateau [23]. Cadenas et al. [24] reported 5 out of 77 Kathmandu males carried dropout of the AMELY and indicated that the five deletions belonged to the same lineage (J2b2-M241) with the age of 6,500±3,300 years of the deletion mutation. The other nulls may represent independent events happening sporadically elsewhere, but may also involve similar mechanisms to the J2's chromosomes.

Conclusion

Our study suggested that the sex test based on AMEL gene alone could lead to mistake for gender identification in some cases. Therefore, Y-STR markers should be required as supplementary determination of gender identification, but Y-STR null haplotypes should be given more attention in the forensic interpretation of mixed evidence to refrain from falsely excluding the suspected assailant. Moreover, these specific Y-STR haplotypes in our study could also give a clue about the deletion pattern of Y chromosome in forensic cases.

Acknowledgments This work was supported by the National Natural Science Foundation of China (81072510) and by the Five-twelfth National Science and Technology Support Program of China.

Conflict of interest The authors declare that they have no conflict of interest.

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The experiments comply with the current laws of the country in which they were performed.