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Resolving relationship tests that show ambiguous STR results using autosomal SNPs as supplementary markers

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Abstract

When using a standard battery of STRs for relationship testing a small proportion of analyses can give ambiguous results – where the claimed relationship cannot be confirmed by a high enough paternity index or excluded with fully incompatible genotypes. The majority of such cases arise from unknowingly testing a brother of the true father and observing only a small number of exclusions that can each be interpreted as one- or two-step mutations. Although adding extra STRs might resolve a proportion of cases, there are few properly validated extra STRs available, while the commonly added hypervariable SE33 locus is four times more mutable than average, increasing the risk of ambiguous results. We have found SNPs in large multiplexes are much more informative for both low initial probabilities or ambiguous exclusions and at the same time provide a more reliable genotyping approach for the highly degraded DNA encountered in many identification cases. Eight relationship cases are outlined where the addition of SNP data resolved analyses that had remained ambiguous even with extended STR typing. In addition we have made simulations to ascertain the frequency of failing to obtain exclusions or conclusive probabilities of paternity with different marker sets when a brother of the true father is tested. Results indicate that SNPs are statistically more efficient than STRs in resolving cases that distinguish first-degree relatives in deficient pedigrees.

Keywords: Paternity testing; STR analysis; SNP; Single nucleotide polymorphism; Forensic DNA typing

1. Introduction

Most laboratories performing relationship testing will rely on the core forensic sixteen-marker short tandem repeat (STR) sets to obtain an exclusion or strong probability of paternity (i.e. reaching virtual proof). However a small proportion of cases show ambiguous results where the claimed relationship cannot be confirmed by a high enough probability or when an exclusion is suggested by just one or two loci. A large proportion of ambiguous results arise from unknowingly testing a first-degree relative of the true father, usually a brother, so the exclusion rate is markedly reduced and a paternity index using a likelihood ratio against a random man does not apply. Less frequently, ambiguous STR results occur from observing exclusions that may originate from germ-line step mutations [1,2]. Step mutations are characterized by one or two repeat additions or diminutions creating an incompatibility that is impossible to distinguish as a mutation or an exclusion. Ambiguous genotypes are particularly difficult to interpret when a brother of the true father is unknowingly tested, as this reduces the total excluding loci. The main recourse for laboratories finding such results is addition of extra STRs to improve the probability or provide clear, unambiguous exclusions. However outside of the principal commercial kits few additional autosomal STRs are validated and readily applicable. Another source of ambiguity is second order exclusions created when primer binding site substitutions lead to the dropout of an amplifiable allele in both parent and offspring. This phenomenon is observed more frequently in certain STRs [3] and the normal approach is to use complimentary marker sets testing identical loci with alternative primer designs [4–6].

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For relationship testing we use extended STR sets comprising 17 markers in two complementary kits: Identifiler® and Powerplex® 16 plus singleplex STRs: D1S1656, D12S391, D18S535 and SE33. Supplementary genotyping has been developed in-house, with three STRs extensively characterized during their initial forensic optimization [7–9]. This choice of kits plus standalone STRs benefits from using 13 loci common to each marker set with different primer sites to help detect dropout, plus eight unique STRs providing powerful extra discrimination. In the past three years we have added single nucleotide polymorphisms (SNPs) to STR analysis in an increasing proportion of complex or deficient relationship tests. Although SNPs have a much lower discriminatory power per locus than STRs, we have used a standardized forensic 52plex assay [10] that matches or exceeds the discriminatory power of 15 STRs. Notably SNPs applied to relationship testing offer a much lower overall mutation rate, typically: $\mu = 2.5 \times 10^{-8}$ compared with $\mu = 10^{-3}$ to 10^{-4} in STRs but the 52plex has provided an ideal complementary approach for three additional reasons: (i) the genomic positions of the 52 SNPs are well spaced, both as a set and in relation to common STRs, to facilitate segregation between related individuals; (ii) SNPs, as binary polymorphisms, are more likely than multi-allelic STRs to show informative second order exclusions in deficient cases (i.e. lacking all pedigree members) and; (iii) the 52plex amplified fragments are all less than 120 bp offering greater success than standard STRs with highly degraded DNA [10–12]. Since a small but consistent proportion of relationship tests we perform involve analysis of human remains, this last characteristic of SNPs provides an important way to avoid a further source of ambiguous results with STRs: uninformative paternity probabilities resulting from incomplete profiles commonly obtained from degraded DNA.

We outline eight cases that failed to give a clear, unequivocal indication of the claimed relationship with STRs alone. Each one showed that adding SNPs improved the paternity index or successfully resolved ambiguous STR exclusions.

2. Materials and methods

2.1. Marker sets used

Table 1 outlines the 21 STRs used, based on two commercial STR mutliplexes: Identifiler® (Applied Biosystems, Foster City, CA) and Powerplex® 16 (Promega, Madison, WI) providing complementary primer set analysis of 13 loci and two specific to each set plus supplementary singleplex STRs: D1S1656, D12S391, D18S535 and SE33. SNP analysis was based on the well-established SNP*for*ID 52plex assay previously described [10, supplementary data at: http://www.snpforid.org/publications.html] and shown to be informative for forensic identification [10–13].

2.2. Statistical analysis

All STR and SNP genotypes were compared amongst tested individuals using *Familias* pedigree analysis software [14] and locally derived (NW Spain) allele frequencies (SNP data in the SNP*for*ID frequency browser: http://spsmart.cesga.es/snpforid.php). In all cases where a paternity index is given as a % probability (*W*) an a priori value of 0.5 was always used. The *Familias* program specializes in suggesting the most likely relationship given the genotypes of tested individuals by calculating the probability of given sets of possible pedigrees. When second order exclusions and step mutations are observed *Familias* is able to factor in specific mutation rates for the loci to compile a probability of the defined relationships. We added values for μ reported in STRbase [1] and listed in Table 1, with range: $\mu = 0.0001$ for TPOX/TH01 to $\mu = 0.0064$ for SE33, with D1S1656, D12S391, D18S535 using an average value of 0.0016 in the absence of current estimates. A universal SNP mutation rate of $\mu = 2.5 \times 10^{-8}$ was used – to date the 52plex SNPs have been validated in trios and extended families without detecting second order incompatibilities for nearly all the SNPs [13,15].

Table 1
STRs sets used and their reported % mutation rates (μ)

Identifiler®			Powerplex® 16			Supplementary STRs		
No.	STR	μ	No.	STR	μ	No.	STR	μ
1	CSF1PO	0.16		CSF1PO		18	**SE33**	0.64
2	**D2S1338**	0.12	16	**Penta D**	0.14	19	**D1S1656**	0.16*
3	D3S1358	0.12		D3S1358		20	**D12S391**	0.16*
4	D5S818	0.11		D5S818		21	**D18S535**	0.16*
5	D7S820	0.1		D7S820				
6	D8S1179	0.14		D8S1179				
7	D13S317	0.14		D13S317				
8	D16S539	0.11		D16S539				
9	D18S51	0.22		D18S51				
10	**D19S433**	0.11	17	**Penta E**	0.16			
11	D21S11	0.19		D21S11				
12	FGA	0.28		FGA				
13	TH01	0.01		TH01				
14	TPOX	0.01		TPOX				
15	vWA	0.17		vWA				

Values of μ with * denote an average rate used in the absence of current estimates. STRs in bold show non-complimentary markers analyzed using single primer pairs.

2.3. Simulation of testing a first-degree relative of the true father

We developed a computer program in R (http://www.r-project.org/) to assess the probability $P(B)$, that a paternal first-degree relative (simplified here to '*brother*' but applicable to the father or a son of the true father) has been tested and is fully compatible with paternity for different combinations of markers, such as 21 STRs or STRs plus 52 SNPs. $P(B)$, for loci: $i...n$, can be defined as:

$$P(B) = \prod_{i}^{l} P(B_i)$$

for each locus : $P(B_i)$

$$= P(B_i|C1)P(C1) + P(B_i|C2)P(C2) + P(B_i|C3)P(C3)$$

where $C1$ = two alleles shared by the true father and a brother, so the probability of $C1$: $P(C1) = 0.25$; $C2$ = one allele shared, $P(C2) = 0.5$; $C3$ = no alleles shared, $P(C3) = 0.25$, and $P(B_i|C1)$, $P(B_i|C2)$ and $P(B_i|C3)$ are calculated from the allele frequencies and mutation rates for each locus i. This allowed estimation of the expected proportion of cases where no exclusions are detected in a brother. Additionally we simulated child–father–brother pedigrees to estimate the paternity index considering two exclusive hypotheses: a brother being the true father against a random man being the true father. More details of the algorithms are available on request.

2.4. Relationship tests examined

The eight cases showing ambiguous STR results can be categorized: (i) a simple disputed paternity trio: 44p06; (ii) paternity analysis of aged, degraded skeletal remains: 70p06 and 20p07; (iii) sibship analysis differentiating half from full sibs: 24p07 and 28p07; (iv) a sib versus paternity counter-claim (individual A claims to be the son of B, B claims to be the half sib of A): 45p06; (v) testing of a sib as proxy for the deceased claimed father: 39p04 and 123p04. With the exception of simple trio 44p06, all families analyzed were deficient, i.e. lacking the mother or the supposed father. Fig. 1 gives the explanatory pedigrees showing alternative relationships analyzed for 44p06 plus the two most complex cases: 123p04, and 45p06.

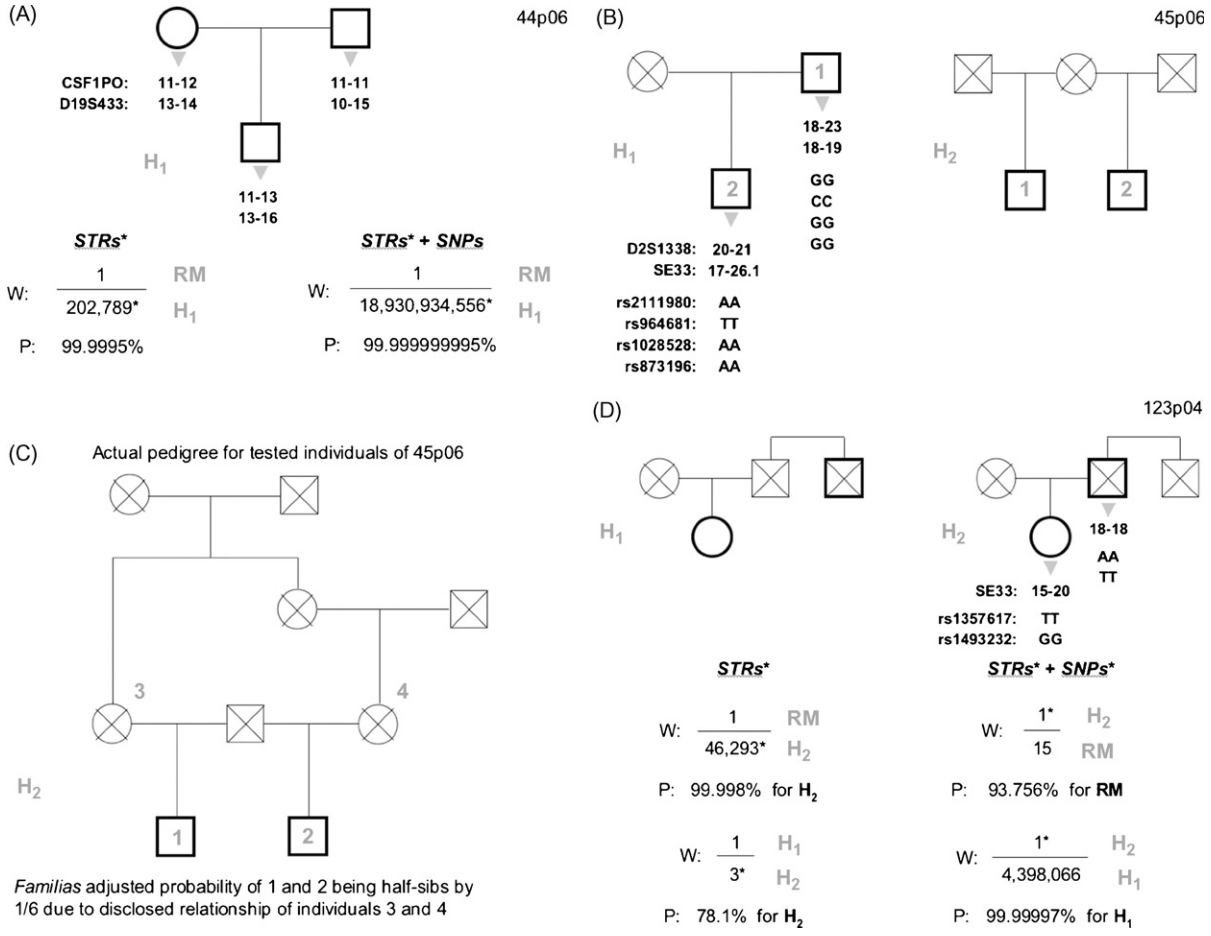

Fig. 1. Three cases showing ambiguous exclusions with likelihood ratios (W) and probabilities (P) for the most likely relationships (hypotheses: H_1, H_2 or RM = random man) using 21 STRs on the left and with the addition of 52 SNPs on the right. Bold pedigree components denote the tested individuals. Likelihoods marked with * were calculated adding mutation rates for excluding loci. Panel C shows the actual pedigree for 45p06 (previously summarized in panel B) disclosed by the family during testing.

3. Results

Results can be divided into two groups: (i) three cases showing ambiguous STR exclusions resolved by adding SNPs, (ii) five cases with uninformative paternity indices improved by adding SNPs, two due to partial STR profiles obtained from degraded bones that gave near-complete SNP profiles in parallel genotyping.

3.1. Cases with ambiguous exclusions

Cases 44p06 and 45p06 (Fig. 1A and B respectively) showed an interesting contrast in their final interpretations although both gave two 1- or 2-step genotype differences after typing 21 STRs. In simple trio 44p06 these comprised a maternal one-step or paternal two-step incompatibility in CSF1PO plus a maternal two-step or paternal one-step incompatibility in D19S433. A reasonable interpretation at this stage would be that two independent mutations are highly unlikely so the tested man is excluded although he may be closely related to the true father. A high paternity index when factoring in the mutation rates also suggested that a brother of the true father could have been tested, but this case remained ambiguous because the incompatibilities were each one- or two-step differences. The addition of SNPs resolved the case since the final paternity index from STRs and SNPs combined with mutation rates, reached 99.999999995% with a predicted probability of failing to exclude a brother of 0.00017 (final row, Table 3).

The sib versus paternity counter-claim case 45p06 had a deficient pedigree: compromising the ability to unambiguously exclude the tested man, while the alternative possibility that the tested men were half-sibs also reduced the excluding power. Additionally the excluding STRs showed one- or two-step differences possible from *either* paternal allele in both D2S1338 and SE33. Adding SNPs provided four independent second order exclusions emphasizing the enhanced ability to resolve deficiency cases provided by binary markers. In fact this case proved to be more challenging than originally supposed, as the true pedigree disclosed by the family showed one man was the offspring of the others aunt (Fig. 1C), with *Familias* allowing a straightforward adjustment to the probability estimates.

Case 123p04, outlined in Fig. 1D, was a fully deficient pedigree (both parents deceased) testing the brother of the deceased man. The tested man claimed paternity of the sole offspring (a daughter, precluding mitochondrial and Y-chromosome analysis). STR analysis gave a single two-step incompatibility in SE33. Factoring in the mutation rate of SE33 gave a probability of paternity against a random man of 99.9978%, but more significantly paternity for the tested man was three times more likely than for the deceased. As SE33 has a mutation rate four times higher than average but the probabilities were not considered strongly indicative of paternity this case remained ambiguous. Addition of SNPs provided two further exclusions of the tested man and, more importantly for resolving the case, when conservative mutation rates of $\mu = 0.00001$ were included for each SNP *Familias* gave a 99.99997% probability in favour of paternity for the deceased man against the brother.

3.2. Cases with uninformative probabilities for the claimed relationship

Table 2 outlines the five cases where SNP analysis provided a significant improvement in the probability of the claimed relationship. The severely degraded skeletal remains tested in cases 20p07 and 70p06 involved respectively: a 35-year-old femur where 9 of 17 STRs were successfully typed and a 10-year-old doubly degraded femur [16] where all 17 STRs failed. SNP profiles detecting 51/52 loci were obtained in both cases [12]. Case 39p04 was identical in structure to 123p04 described above and in Fig. 1D, but here addition of SNPs provided a strong indication that the tested brother was the true father by increasing the paternity index 35-fold to 99.994% against the deceased man.

Table 2
Five cases testing three different sets of alternative pedigrees

Case	Test	Relationship hypothesis			STRs	STRs + SNPs
			H_1	H_2	$W = H_1/H_2$ %P	$W = H_1/H_2$
28p07	Sib analysis		Half-sib	Full-sib	W: 1/3 P: 75%	1/1,193 99.91%
24p07	Sib analysis				1/897 99.89%	1/12,140,628,977 99.999999%
		RM	H_1		$W = RM/H_1$	$W = RM/H_1$
20p07	Identification of remains (paternity)	Random man is father	Tested man is father		1/139* 98.28%	1/58,823‡ 99.9983%
20p06	Identification of remains (paternity)				No profile	1/14,286‡ 99.993%
		RM	H_1	H_2	$W = RM/H_2$	$W = RM/H_2$
39p04	Brother of deceased man tested as proxy	Random man is father	Deceased man is father	Brother of deceased is father	1/11,156,811 99.99999%	1/353, 340,169 99.99999999%
					$W = H_1/H_2$	$W = H_1/H_2$
					1/496 99.799%	1/17,178 99.994%

RM (random man), H_1 and H_2 relationship hypotheses were assessed with likelihood ratios (W) and % probabilities (P) for 21 STRs alone and STRs plus 52 SNPs. Values marked with a suffix denote partial profiles: * = 9/17 STRs, ‡ = 51/52 SNPs.

Table 3
Predicted probabilities of a brother of the true father being compatible with paternity (no exclusions detected) for different marker sets and their combinations

Marker set (number of loci)	Probability of no detected exclusions in a brother (standard deviation in brackets)	Proportion of PI values higher than 1 (%)
Identifiler® (15)	0.02657 (0.011)	6.9
MiniFiler® (8)	0.12044 (0.035)	11.9
Powerplex® 16 (15)	0.02503 (0.01)	6.4
Profiler Plus® (9)	0.09648 (0.029)	10.7
Identifiler® + Powerplex® 16 (17)	0.01395 (0.006)	5.0
17 core STRs + 4 supplementary	0.00277 (0.001)	2.4
SNP*for*ID ID-SNPs (52)	0.05165 (0.018)	6.1
21 STRs + 52 SNPs	0.00017 (0.0001)	0.5

The right column lists the proportion of uninformative PI values that simulations suggest can be expected from each marker set (i.e. a PI value higher than 1, when a brother is more likely than a random man to be the father).

3.3. Probability of failing to exclude first-degree relatives of the true father

We calculated the probability of a brother of the true father showing no exclusions against the tested child. Here an exclusion denotes a Mendelian incompatibility given the hypothesis of the tested man's brother being the true father. Probabilities are shown in Table 3 with the corresponding standard deviations for common STR sets, the 52 ID-SNP set and their combinations. Profiler Plus® and MiniFiler® are included as we now regularly use these in combination with SNPs to analyze degraded DNA when Identifiler® and Powerplex® 16 give incomplete profiles. The values reveal that both the core STR sets have a comparable failed exclusion rate of ~2%, while SNPs alone show a rate of ~5%: indicating that in about 1 in 50 cases using STRs a brother is completely compatible with paternity since no exclusions are detected. The slightly lower power of SNPs compared to STRs can be partly explained because with binary markers heterozygotes (in either brother or true father) are uninformative for both inclusions and exclusions in deficient families. This loss of discrimination power in SNPs is compensated by using a much higher total number of loci compared to STRs. The addition of six STRs to either core set lowers the failed exclusion rate to 1 in 360 but notably the rate is reduced more than 16-fold to 1 in 5880 when 21 STRs and 52 SNPs are combined.

Fig. 2 plots the paternity indices obtained from the simulation of father–brother–child pedigrees. Computation of the paternity index for the alternative hypotheses: paternity of a brother against paternity of a random man provides a more realistic simulation of how an actual paternity case is normally approached when no exclusions are detected. Values for this paternity index higher than one indicate that no exclusions have been detected in the brother so he is more likely than a random man to be the father, a typical ambiguous result. Table 3 lists the proportion of paternity indices higher than one for each marker set. Fig. 2 plots the complete range of PI values obtained for each marker set in 6577 simulations, ranked left to right, from most to least informative, so lower plot lines indicate a higher proportion of informative PI values obtained. Although SNPs give a 'ladder-shape' plot because only opposite homozygotes between brother and child are informative, the overall proportion of highly informative PI values is seen to be

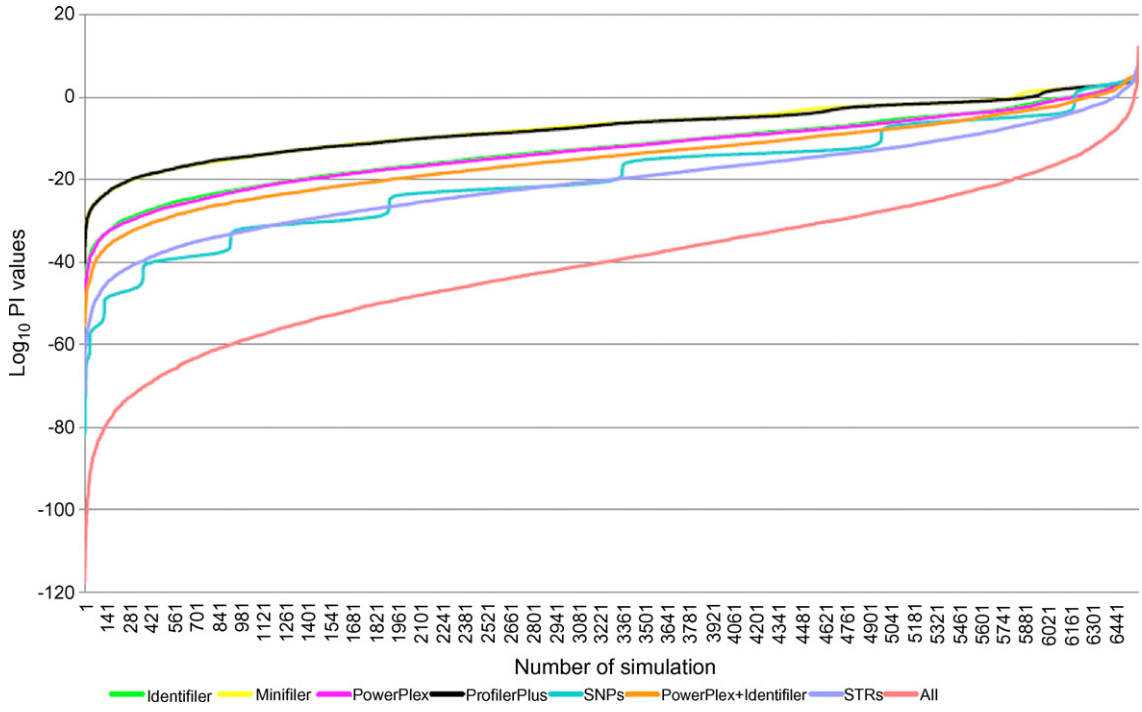

Fig. 2. Range of $\log_{10}$ PI values from 6577 simulations for different marker sets given the two hypotheses: a brother of the true father is compatible with paternity versus a random man. The 'ladder-shape' of the SNP PI values is due to the fact that only opposite homozygotes between brother and child are informative. The plot labelled *STRs* denotes all 21 unique STR loci in combination, the plot labelled *All* denotes 21 STRs plus 52 SNPs.

equivalent to the plot for a full set of 21 STRs. Table 3 shows the proportion of PI values higher than one obtained for Identifiler with 6.9% and Powerplex 16 with 6.4% are both slightly higher than 52 SNPs with 6.1%. Therefore results indicate that SNPs are more efficient than STRs for resolving cases that attempt to distinguish first-degree relatives in deficient pedigrees. Overall Table 3 and Fig. 2 clearly indicate that combining STRs and SNPs provides the most secure interpretative framework for relationship testing of close relatives, reducing to 0.5% the total proportion of ambiguous paternity indices.

4. Discussion

Each of the eight relationship tests reported gave some ambiguity in the STR results that was successfully resolved by including SNP analysis. The SNP profiles were generated from a straightforward multiplex assay optimized and validated for forensic identification, where a very low frequency of incompatibilities in normal trios has already been established [13,15]. These cases clearly illustrate that the addition of 52 SNPs removes the element of doubt involved in the interpretation of challenging relationship tests using extended STR typing. We found the combination of adding a large battery of SNPs and using *Familias* to obtain reliable probabilities for each possible relationship created a more secure framework for interpreting results.

The application of SNPs in relationship testing has not been widespread to date because nearly all paternity cases are adequately resolved with existing well validated STR sets. However a characteristic of SNPs often listed in their favour for relationship testing is a comparatively low mutation rate, suggesting SNPs markedly reduce the risk of ambiguous exclusions arising from mutation. The distinction should be made here between exclusions created by allelic instability and those created by allele dropout from primer binding site mutations. SNPs have a much lower rate of allele mutation than STRs reflected in the rates detailed above. In contrast, SNP analysis of ~50 loci (assuming use of one extension plus two PCR primers and 20 bp average lengths) will be prone to ~5 times more allele dropouts from binding site mutations than 15 STRs. However since the average nucleotide substitution rate is extremely low [16] this has a minor effect on the rate of incompatibilities compared to the meiotic instability of STRs. Additionally, the effect of genotyping 50 or more binary markers makes it most likely that a primer site mutation creates a single second order exclusion contrasting with the overall pattern of results.

There is persuasive evidence in the cases described and previous studies [Fig. 5 of 13] that SNPs can add the extra discrimination power needed to resolve relationship tests that routinely compare closely related individuals. It is likely that this characteristic of SNPs is largely due to the relatively high number of segregations occurring between first-degree relatives with an extensive marker set showing the widest possible genomic distribution. Furthermore the low SNP mutation rate makes the interpretation of any exclusions found amongst closely related individuals much more secure. Applications that can therefore benefit from SNP analysis include disaster victim identification, immigration testing, complex pedigree reconstruction and the analysis of deficient families that forms a large proportion of tests identifying missing persons. The fact that SNPs additionally offer greater success when typing highly degraded DNA indicates that combining SNPs, rather than extra STRs, with the current core markers offers the best way to improve the interpretation of challenging relationship tests in the future.

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