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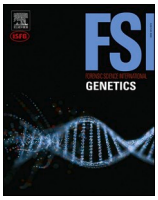

Improving the differentiation of closely related males by RMplex analysis of 30 Y-STRs with high mutation rates

Franz Neuhuber [a], Bettina Dunkelmann [a], Ines Grießner [a], Katharina Helm [a], Manfred Kayser [b,1], Arwin Ralf [b,1,*]

[a] Department of Forensic Medicine and Forensic Neuropsychiatry, University of Salzburg, Salzburg, Austria
[b] Department of Genetic Identification, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands

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ABSTRACT

The discovery of rapidly mutating (RM) Y-STRs started to move the field of forensic Y-STR analysis from male lineage identification towards male individual identification. Previously, the forensic value of RM Y-STRs for differentiating male relatives was limited due to the modest number of 13 identified RM Y-STRs. Recently, new RM Y-STRs were discovered, with strong expectations for significantly improving male relative differentiation; however, empirical evidence is missing yet. More recently, the genotyping method RMplex for efficiently analyzing 30 Y-STRs with high mutation rates, including all 26 currently known RM Y-STRs, was introduced. Here, we applied RMplex as well as the current state-of-the-art commercial Y-STR kit: Yfiler™ Plus PCR Amplification kit, to several hundreds of DNA-confirmed father-son pairs. Newly established estimates confirmed the high mutation rates of novel and previous RM Y-STRs. By combining current with previous data, we provide updated consensus estimates of mutation rates for all 49 Y-STRs targeted with both methods. Based on RMplex, 42% of 499 father-son pairs were differentiated, while 14% of 530 pairs based on Yfiler™ Plus, and 48% of 499 pairs based on both methods combined. Regarding brothers, RMplex also clearly outperformed Yfiler™ Plus, with differentiation rates of 62% and 33%, respectively. By combining both methods 72.9% of the brothers showed at least one mutation. For unrelated males, both methods achieved a discrimination capacity of 99.8% and a haplotype diversity of 0.999991, since all males had different haplotypes, except for two, perhaps indicating a hidden paternal relationship. Overall, this study underlines the value of RM Y-STRs in general and RMplex in particular for differentiating male relatives highly relevant in forensic genetics. It provides the first empirical evidence on the high value of RMplex for differentiating close male relatives, which for father-son pairs was almost 60% higher than with the initial set of 13 RM Y-STRs and three times higher than with Yfiler™ Plus. Based on our results from closely related males, we expect RMplex to also improve the differentiation of more distantly related males significantly, which needs empirical demonstration in future studies. We encourage the forensic community to apply RMplex in all forensic cases where a match with a commercial Y-STR kit was obtained between the male suspect and the evidence material, or to solely use RMplex in such cases, aiming to find out if the male suspect or any of his male paternal relatives left the evidence material at the crime scene.

1. Introduction

Male-specific Y-chromosomal short tandem repeats (Y-STRs) have several applications in forensic genetics: they are used to predict the paternal ancestry of an unknown donor of a crime scene trace, to infer surnames, or for familial searching to trace unknown perpetrators. Until now, however, the most valuable contribution of Y-STRs to the forensic toolbox is their application in unbalanced male-female mixtures, as are often observed in sexual assault cases, where typically standard autosomal STR-profiling is not informative for identifying the male perpetrator. In such cases, Y-STRs are applied to characterize the paternal lineage of the male contributor aiming to identify the male perpetrator [1]. The largest limitation of this application is that typically Y-STR haplotypes are shared between many paternally related [2,3] and

* Corresponding authors.
E-mail address: a.ralf@erasmusmc.nl (A. Ralf).
[1] These authors contributed equally to this work

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sometimes even unrelated males [4–6]. This poses a problem in how to evaluate in court the evidentiary value of a match between the suspect's Y-STR profile (haplotype) and that from the crime scene material, as the sample donor could be the suspect or, with the very same probability, one of his male paternal relatives [7–11]. The number of haplotype matches of unrelated individuals can be significantly reduced by increasing the number of Y-STRs analyzed [12,13]. Nevertheless, the problem of haplotype sharing between many paternally related males remains.

Rapidly mutating Y-STRs (RM Y-STRs) have been proposed as a solution to overcome this problem [14]. Their increased mutation rates (i.e., one or a few mutations per locus per 100 generations) allow to differentiate more paternally related males than possible with Y-STRs characterized by moderate mutation rates (i.e., one or a few mutations per locus per 1000 generations). As haplotypes derived from RM Y-STRs are shared by less relatives compared to haplotypes based on Y-STRs with moderate mutation rates, the evidentiary value of a haplotype match based on RM Y-STRs is higher. However, until recently, the number of known RM Y-STRs was limited to the 13 initially discovered markers [15] and thus was the male relative differentiations rates they can achieve, i.e., 27% for father-sons (one meiosis), 46% for brothers and grandfather-grandson pairs (two meioses), and 62% for cousins (four meioses) [2].

To help facilitating the more efficient differentiation of unrelated and related males available with RM Y-STRs [4], the current generations of widely used commercial Y-STR kits, such as Yfiler™ Plus PCR Amplification kit (Thermo Fisher Scientific) (in the following referred to as Yfiler Plus) or PowerPlex® Y23 System (Promega) include some of the 13 initially identified RM Y-STRs (six and two, respectively). The actual male relative differentiation rates obtainable with such latest generation commercial Y-STR kits has yet to be empirically determined. They are expected to be lower than that obtained for the full set of 13 RM Y-STRs, because most of the 13 markers, including those with the highest mutation rates, were not included in these state-of-the-art commercial Y-STR kits. Nevertheless, the relative differentiation rates obtained with these commercial kits will be higher than those of their predecessors: AmpFLSTR™ Yfiler™ PCR Amplification Kit (in the following referred to as Yfiler), previously established at 5% for father-sons [2], and PowerPlex® Y System, because both previous kits include fewer Y-STR loci and no RM Y-STRs.

Recently, 12 novel RM Y-STRs were identified [16], almost doubling the number of available RM Y-STRs. Empirical male relative differentiation rates obtainable with the full set of currently known 26 RM Y-STRs are not available as of yet. Theoretically expected male differentiation rates previously calculated based on the locus-specific mutation rates inform that this increased RM Y-STR set should allow differentiating father and sons with a rate of approximately 44%, brothers with 69% and cousins (separated by four meioses) with 90% [16]. More recently, a novel genotyping method termed RMplex [17] was introduced for the effective analysis of 30 Y-STRs with high mutation rates, including all currently known 26 RM Y-STRs together with four additional Y-STRs with elevated mutation rates.

In the present study, we apply RMplex for analyzing 30 Y-STRs with high mutations rates to the closest possible, and therefore most challenging to differentiate, paternal male relatives by analyzing 530 DNA-confirmed father-son pairs to empirically quantify mutation rates as well as differentiation rates of father-sons, brothers, and unrelated males. Moreover, we compare these findings to those we obtained with the current state-of-the-art commercial Y-STR kit Yfiler Plus that targets 25 Y-STRs including six RM Y-STRs that overlap with RMplex. Our study is the first to deliver: i) independent confirmation of mutation rates for the recently discovered novel RM Y-STRs, ii) empirical father-son and brother differentiation rates for RMplex and Yfiler Plus, and iii) empirical differentiation of unrelated males with RMplex and Yfiler Plus. Moreover, we provide updated consensus mutation rate estimates for 49 Y-STR included in both methods by combining the data produced in the present study with those from the literature.

2. Material and methods

2.1. DNA samples

A total of 1016 male individuals from Austria with first degree relationships to at least one other male individual in the study were analyzed including 482 fathers with a total of 534 sons. In the cases where a father had more than one son included, each of the sons formed a pair to be analyzed with the same father. Considering father-son pairs with the same father (but different sons) may cause some dependency as the alleles of the father determine the mutations in the son. However, the number of dependent pairs with the same father was with 73 relatively small compared to the number of independent father-son pairs (457); hence no large impact on the results is expected. Amongst the sons were 4 pairs of monozygotic twins of which only one was considered for the consequent analyses and the other was excluded. Moreover, the sample set contained two dizygotic twin pairs, which were considered as brothers. In total, 530 father-son pairs and 92 brother pairs could be defined in the sample set.

All DNA samples come from previous paternity testing using the full mother-father-son/sons settings, where achieved paternity probabilities based on autosomal STRs were $> 99.9999\%$. This allows concluding true biological father-son relationships for all of the pairs included in this study, which serves as key prerequisite for mutation rate and father-son differentiation rate analysis. The samples were fully anonymized and only the father-son relationships (and the brother relationships by extension) were conserved. The ethics committee of the University of Salzburg approved this study (EK-GZ: 07/2021).

2.2. Genotyping

RMplex is a genotyping assay that consists of two non-overlapping sets of Y-STRs of which the results are analyzed combined. The total of 30 Y-STRs contain various multi-copy markers leading to a total of 44 loci being amplified. Genomic DNA was extracted using the chelex 100 extraction method. The exact concentrations were unknown; however, these samples were previously used for paternity testing, here the same amounts of DNA were used. PCR amplifications were performed under the same conditions as described previously [17]. For DYS570 the alternative forward primer that was suggested in the original method publication [17] was used. The only deviation from the original protocol was that here the amplification was performed in a reduced reaction volume of 10 μL. The DNA was amplified in PE 9700 Thermal Cycler (Perkin Elmer). The resulting amplification products were separated using a 3500 Genetic Analyzer (Thermo Fisher Scientific); and the resulting electropherograms were analyzed using Genemapper ID-X, Version 1.4. For Yfiler Plus, the same instruments were used, the manufacturer's recommendations were followed, except for the PCR reaction volume which was reduced to 12.5 μL. For both methods, allele calling was done by two experienced analysts independently, and in case of conflicting outcomes a third expert was involved for clarification.

2.3. Data analysis

Mutation rates and male relative differentiation rates were calculated using the frequentist approach. On top of the data generated here, mutations rate data was also obtained from literature, for a fair comparison, the reference mutation rates were recalculated using the frequentist approach in case the original publication used a different (i.e., Bayesian) approach. The 95% confidence intervals of the mutation and male relative differentiation rates were calculated using the Clopper–Pearson interval [18]. Different mutation rates estimates were compared to each other using Fisher's exact tests [19]; Bonferroni correction [20] was applied to account for multiple testing. For the

mutation rate estimates, all pairs were used for analysis, in case of partial profiles the pair was excluded for the analysis of only the specific Y-STRs that had missing data. For the male relative differentiation rate and the haplotype diversity estimations, pairs and individuals, respectively, with missing data for a single or more Y-STRs were excluded from the analysis.

3. Results and discussion

3.1. Assay performances

All DNA samples were genotyped using three assays: the two multiplex assays of the non-commercial RMplex and the single multiplex assay of the commercial Yfiler Plus kit. While Yfiler Plus delivered complete Y-STR profiles for all 1016 individual DNA samples from all 530 father-son pairs, RMplex achieved complete Y-STR profiles for 983 (97%) individual samples from 499 (94%) of the 530 pairs. A total of 31 (3.1%) out of the 1016 samples showed at least one locus dropout with RMplex 1, while with RMplex 2 locus-dropouts were seen in six (0.6%) samples. Locus-dropouts with RMplex were mostly caused by three Y-STRs i.e., DYR88, DYS1013, and DYF404S1, which delivered no result in 19 (1.9%), 13 (1.3%) and 9 (0.9%) of the 1016 individual DNA samples, respectively. Notably, these same Y-STRs also showed a reduced performance in the initial RMplex validation study [17]. Overall, the technical performance of Yfiler Plus was superior to that of RMplex this may in part be explained by RMplex being less sensitive than Yfiler Plus [17,21]. Another reason may be that RMplex contains Y-STRs with larger PCR fragment sizes than the longest ones of Yfiler Plus and

Table 1
Empirically established locus-specific mutation rates obtained for 49 Y-STRs based on RMplex and Yfiler Plus data from a total of 530 DNA-confirmed father-son pairs.

Marker	Assay	Total pairs	Mutations	Expansions	Contractions	Mutation rate (x 10^{-3})	95% confidence interval (x 10^{-3})	Reference mutation rate (x 10^{-3})[#]	p-value
DYF399S1	RMplex	530	41	14	27	77.4	56.1–103.5	77.5	1.0000
DYS724	RMplex	529	28	14	14	52.9	35.5–75.6	46.4	0.5583
DYS712	RMplex	530	23	8	15	43.4	27.7–64.4	27.2	0.0852
DYF1001	RMplex	528	19	13	6	36.0	21.8–55.6	52.0	0.1593
DYF1000	RMplex	530	19	15	4	35.8	21.7–55.4	35.9	1.0000
DYF403S1a	RMplex	530	15	8	7	28.3	15.9–46.3	30.6	0.8828
DYS711	RMplex	530	14	5	9	26.4	14.5–43.9	26.6	1.0000
DYS1007	RMplex	530	12	6	6	22.6	11.8–39.2	15.5	0.2553
DYS449	Yfiler Plus+RMplex	530	11	9	2	20.8	10.4–36.8	11.8	0.1363
DYR88	RMplex	511	9	3	6	17.6	8.1–33.2	29.1	0.2039
DYS713	RMplex	529	9	4	5	17.0	7.8–32.0	14.2	0.6797
DYS612	RMplex	530	8	3	5	15.1	6.5–29.5	14.1	0.8366
DYS1013	RMplex	517	7	5	2	13.5	5.5–27.7	9.9	0.4683
DYS1010	RMplex	527	7	2	5	13.3	5.4–27.2	14.2	1.0000
DYS526b	RMplex	528	7	3	4	13.3	5.3–27.1	12.1	0.8225
DYS458	Yfiler Plus	530	7	3	4	13.2	5.3–27.0	8.0	0.2975
DYF1002	RMplex	530	7	3	4	13.2	5.3–27.0	17.9	0.5614
DYF403S1b	RMplex	529	6	2	4	11.3	4.2–24.5	11.4	1.0000
DYS570	Yfiler Plus+RMplex	530	6	2	4	11.3	4.2–24.5	11.9	1.0000
DYS385	Yfiler Plus	530	6	1	5	11.3	4.2–24.5	5.1	0.1286
DYF387S1	Yfiler Plus+RMplex	530	6	3	3	11.3	4.2–24.5	15.5	0.6795
DYS1003	RMplex	530	6	2	4	11.3	4.2–24.5	13.0	1.0000
DYS1005	RMplex	530	6	3	3	11.3	4.2–24.5	9.3	0.6195
DYS576	Yfiler Plus+RMplex	530	5	3	2	9.4	3.1–21.9	13.9	0.5142
DYS533	Yfiler Plus	530	5	2	3	9.4	3.1–21.9	4.6	0.1988
DYS460	Yfiler Plus	530	4	2	2	7.5	2.1–19.2	5.8	0.7512
DYF404S1	RMplex	520	3	2	1	5.8	1.2–16.8	12.1	0.4815
DYS547	RMplex	529	3	2	1	5.7	1.2–16.5	23.2	0.0093
DYS456	Yfiler Plus	530	3	2	1	5.7	1.2–16.5	4.6	0.7242
DYS393	Yfiler Plus	530	3	2	1	5.7	1.2–16.5	1.7	0.1424
DYS439	Yfiler Plus	530	3	1	2	5.7	1.2–16.5	3.5	0.4446
DYS481	Yfiler Plus	530	3	2	1	5.7	1.2–16.5	4.6	0.7253
DYS1012	RMplex	530	3	2	1	5.7	1.2–16.5	19.2	0.0276
DYS626	RMplex	529	2	1	1	3.8	0.5–13.6	11.8	0.1317
DYS627	Yfiler Plus+RMplex	530	2	2	0	3.8	0.5–13.6	11.9	0.1342
DYS19	Yfiler Plus	530	2	0	2	3.8	0.5–13.6	4.0	1.0000
YGATAH4	Yfiler Plus	530	2	1	1	3.8	0.5–13.6	2.8	0.6665
DYS391	Yfiler Plus	530	2	1	1	3.8	0.5–13.6	2.8	0.6660
DYS518	Yfiler Plus+RMplex	530	2	1	1	3.8	0.5–13.6	18.0	0.0180
DYS437	Yfiler Plus	530	2	0	2	3.8	0.5–13.6	1.1	0.2307
DYF393S1	RMplex	530	2	0	2	3.8	0.5–13.6	8.2	0.3869
DYS442	RMplex	528	1	0	1	1.9	0–10.5	9.4	0.1356
DYS389I	Yfiler Plus	530	1	1	0	1.9	0–10.5	5.1	0.4695
DYS448	Yfiler Plus	530	1	0	1	1.9	0–10.5	0.0	0.2328
DYS635	Yfiler Plus	530	0	0	0	0.0	0–6.9	3.5	0.3463
DYS389II	Yfiler Plus	530	0	0	0	0.0	0–6.9	3.4	0.3464
DYS390	Yfiler Plus	530	0	0	0	0.0	0–6.9	1.1	1.0000
DYS438	Yfiler Plus	530	0	0	0	0.0	0–6.9	0.6	1.0000
DYS392	Yfiler Plus	530	0	0	0	0.0	0–6.9	0.6	1.0000

[#] Reference mutation rates were those combined from Ballantyne et al., 2010 [15] and Ralf et al., 2020 [16].

therefore is more prone to reduced performance caused by low DNA quantity and/or quality. Lastly, Yfiler Plus may be more resilient against PCR inhibitors. In general, it is not unexpected to see that genotyping assays developed by academia, such as RMplex, show reduced performance compared to those developed by industry, such as Yfiler Plus, as commercial companies typically spend more resources on the assay development than are available to academia. Of the six Y-STRs that overlap between Yfiler Plus and RMplex, all obtained results were fully concordant between these two genotyping methods.

3.2. Mutation analysis

In total, we identified 333 Y-STR mutations among the 530 father-son pairs analyzed, of which 289 were detected with RMplex and 76 with Yfiler Plus including 32 mutations at the six RM Y-STRs targeted by both methods. Among the 333 Y-STR mutations observed, 325 were single-step repeat mutations (97%) and 9 were multi-step repeat mutations (3%). These results, showing a vast excess of single-step over multi-step repeat mutations, are in line with those from previous studies of Y-STR mutations in father-son pairs [15,16]. Moreover, we observed slightly more repeat contractions: 175 (53%) than repeat expansions: 158 (44%); but overall, the number of contractions and expansions were very similar, which also agrees with previous findings [15,16]. All observed mutations with the genotype of both father and son are shown in supplementary Table S1.

On average, RMplex detected a mean number of 0.54 mutations per pair with a standard deviation of 0.73 and a range of 0–3 mutations per pair. In contrast, Yfiler Plus detected a mean number of 0.14 mutations per father-son pair with a standard deviation of 0.38 and a range from 0 to 3 mutations per pair. When combining both methods and using all 49 Y-STRs together, a mean number of 0.69 mutations per pair was found with a standard deviation of 0.89 and ranging from 0 to 4 mutations per pair.

We estimated locus-specific mutation rates for all 49 Y-STRs targeted with both methods (Table 1) and compared these newly estimated mutations rates with those previously reported in father-son based studies for the same Y-STRs [15,16]. Based on Fisher's exact test (see Table 1 for the p-values), three of the 49 Y-STRs showed p-values below the 0.05 nominal significance threshold, which were DYS547 (p-value 0.0093), DYS1012 (p-value 0.0276), and DYS518 (p-value 0.0180). For these three Y-STRs, the mutation rate estimates in the present study were lower than described in the reference literature. However, when correcting for multiple testing using Bonferroni correction with an adjusted significance threshold of 0.0010, none of the Y-STRs showed a significant mutation rate difference. We also found Y-STRs displaying higher mutation rate estimates than described in literature, e.g., DYS712, DYS1007, and DYS449, albeit none of those showed a statistically significant difference.

These observed mutation rate differences, none being statistically significant after multiple testing correction, may be related to stochastic effects caused by small sample size, given that Y-STR mutations being relatively rare events even for those with increased mutation rates. Since approximately three fold more father-son pairs were included in the previous mutation rate studies [15,16] compared to the present one, it may be expected that the previously obtained mutation rate estimates are closer to the ground truth than those reported here. Nevertheless, it should be noted that mutation rates estimates can show clear differences between studies; hence, the most conservative approach may be to combine data from multiple studies.

To this end, we carried out an extensive, yet not exhaustive, literature search for published Y-STR mutation data based on father-son pair analysis involving the 49 Y-STRs targeted here with both methods. We pooled the data of the present study with those obtained from 31 previous studies [2–4,15,16,22–47] for the same loci (Supplementary Table S2), covering a total ranging from 2025 to 12,387 father-son pairs depending on the Y-STR marker. The newly established updated locus-specific consensus mutation rate estimates are presented in Table 2, and could serve as a new reference for future studies.

Moreover, using these updated consensus mutation rates, we revisited the four-category classification system that we previously proposed [16] to classify the 49 Y-STRs (Table 2). Twenty-four Y-STRs were classified as RM Y-STRs (mutation rates $> 1 \times 10^{-2}$), of which previously 23 were described as such [15,16] and one (DYS1013) as fast mutating (FM) Y-STR [16]. Nine Y-STRs were classified as FM Y-STRs (mutation rates $5 \times 10^{-3} - 1 \times 10^{-2}$), of which previously five were previously descripted as such, three as RM Y-STRs (DYS403S1b, DYS626, and DYS570) [15], and one (DYS389II) as moderately mutating (MM) Y-STR. Thirteen Y-STRs were classified as MM Y-STRs (mutation rate $1 \times 10^{-3} - 5 \times 10^{-3}$), of which previously 11 were described as such and two DYS460 and DYS389I) as FM- Y-STRs [15]. The remaining three Y-STRs (DYS448, DYS392, and DYS438) were classified as slowly mutating (SM) Y-STRs (mutation rate $< 10^{-3}$) and were previously described as such [15]. According to this revisited classification, RMplex contains 24 RM Y-STRs (80%) and six FM Y-STRs (20%), whereas Yfiler Plus includes five RM Y-STRs (20%), four FM Y-STRs (16%), 13 MM Y-STRs (52%) and three SM Y-STRs (12%).

The differences between the current classification and that reported previously may reflect uncertainties in the mutation rate estimates and the difficulty of categorizing Y-STRs by using sharp mutation rate borders. For Y-STRs with mutation rates close to the defined borders, a slight increase of the updated mutation rate results in a classification upgrade (e.g. DYS1013), while a slight decrease in a downgrade (e.g. DYS1005). However, by increasing the sample size of the mutation rate underlying father-son pairs further and further, the consensus estimates will become more and more robust, decreasing fluctuations in the next updated mutation rate estimates and thus classification changes. As previously emphasized [16], it is our opinion that the four-category classification system does provide a practically useful way to group (Y-)STRs based on their mutability.

In general, differences in mutation rates between studies done in different populations could also reflect biological differences of these populations, which e.g., can be linked to differences in haplogroup compositions of the populations [48]. To determine if such effects could be seen for the 49 Y-STRs that were analyzed in the current study, we established separate datasets of father-son pair based mutation data for the two major populations for which most mutation data were available in the literature and including the present data, namely for Europeans and for Asians (Table 2). For the most recently discovered Y-STRs, mutation rate data from Asian populations are not yet available; hence, these markers had to be excluded from this analysis, leaving a total of 35 Y-STRs in this analysis. Comparing mutation rate estimates obtained from Europeans and Asians using Fisher's exact test revealed three of the 35 Y-STRs tested with p-values below the 5% nominal significance threshold: DYF399S1 (p-value 0.0011), DYS570 (p-value 0.0177), and DYS19 (p-value 0.0417). For all three markers, the mutation rate estimates based on the European males were higher than those from Asian males. However, after Bonferroni correction for multiple testing, none of the differences remained statistically significant. Despite not being statistically significant, we noted Y-STRs with over two-fold differences in mutation rate estimates between these two major populations, e.g., DYS390, DYS389I, DYS19, and DYS392 (Table 2), which are all Y-STRs with lower mutation rates. It is not surprising that larger differences are most common in Y-STRs with lower mutation rates, where due to low numbers of mutational events observed, stochastic effects have a large impact. Notably, the observed mutation rate differences could lead to different classifications of several Y-STRs, e.g., DYF387S1, DYS403S1b, DYS570 would be classified as RM Y-STRs based on European data, while based on the Asian data they would be FM Y-STRs. More data for different major populations are needed to get better insights into population effects on Y-STR mutation rates. If in the future, statistically significant differences in Y-STR mutation rates between major populations based on large-enough sample size would be established, it may

Table 2
Updated consensus estimates of locus-specific mutation rates for 49 Y-STRs by combining current data with literature data from father-son pair analyses.

	Overall consensus					Europe			Asia			
	Total pairs	Mutations	Mutation rate (x 10^{-3})	95% C.I. (x 10^{-3})	Classification	Total pairs	Mutations	Mutation rate (x 10^{-3})	Total pairs	Mutations	Mutation rate (x 10^{-3})	p-value
DYF399S1	7655	481	62.8	57.5–68.5	RM Y-STR	2324	180	77.5	4320	244	56.5	0.0011
DYF1001	2144	103	48.0	39.4–58.0	RM Y-STR	2144	103	48.0	n.a.	n.a.	n.a.	n.a.
DYS724	2145	103	48.0	39.4–57.9	RM Y-STR	2145	103	48.0	n.a.	n.a.	n.a.	n.a.
DYF1000	2146	77	35.9	28.4–44.6	RM Y-STR	2146	77	35.9	n.a.	n.a.	n.a.	n.a.
DYS712	2476	77	31.1	24.6–38.7	RM Y-STR	2146	67	31.2	330	10	30.3	1.0000
DYF403S1a	7265	198	27.3	23.6–31.3	RM Y-STR	2034	61	30.0	4320	117	27.1	0.5151
DYS711	2146	57	26.6	20.2–34.3	RM Y-STR	2146	57	26.6	n.a.	n.a.	n.a.	n.a.
DYR88	2127	56	26.3	19.9–34.1	RM Y-STR	2127	56	26.3	n.a.	n.a.	n.a.	n.a.
DYS1007	2146	37	17.2	12.2–23.7	RM Y-STR	2146	37	17.2	n.a.	n.a.	n.a.	n.a.
DYF1002	2146	36	16.8	11.8–23.1	RM Y-STR	2146	36	16.8	n.a.	n.a.	n.a.	n.a.
DYS612	8360	136	16.3	13.7–19.2	RM Y-STR	2668	39	14.6	4320	71	16.4	0.6211
DYS1012	2146	34	15.8	11.0–22.1	RM Y-STR	2146	34	15.8	n.a.	n.a.	n.a.	n.a.
DYS547	7900	116	14.7	12.1–17.6	RM Y-STR	2208	42	19.0	4320	57	13.2	0.0861
DYS627	11871	172	14.5	12.4–16.8	RM Y-STR	2667	35	13.1	7832	115	14.7	0.6367
DYS1010	2143	30	14.0	9.5–19.9	RM Y-STR	2143	30	14.0	n.a.	n.a.	n.a.	n.a.
DYS713	3752	52	13.9	10.4–18.1	RM Y-STR	2145	32	14.9	1607	20	12.4	0.5741
DYS518	11288	150	13.3	11.3–15.6	RM Y-STR	2086	30	14.4	7830	91	11.6	0.3126
DYS576	12387	157	12.7	10.8–14.8	RM Y-STR	2897	35	12.1	7762	101	13.0	0.7731
DYS1003	2146	27	12.6	8.3–18.3	RM Y-STR	2146	27	12.6	n.a.	n.a.	n.a.	n.a.
DYF404S1	8312	104	12.5	10.2–15.1	RM Y-STR	2259	24	10.6	5042	65	12.9	0.4889
DYS526b	7871	97	12.3	10.0–15.0	RM Y-STR	2179	27	12.4	4320	52	12.0	0.9049
DYS449	12303	138	11.2	9.4–13.2	RM Y-STR	2518	37	14.7	8413	85	10.1	0.0653
DYS1013	2133	23	10.8	6.8–16.1	RM Y-STR	2133	23	10.8	n.a.	n.a.	n.a.	n.a.
DYF387S1	11150	114	10.2	8.4–12.3	RM Y-STR	2334	34	14.6	7805	75	9.6	0.0510
DYS1005	2146	21	9.8	6.1–14.9	FM Y-STR	2146	21	9.8	n.a.	n.a.	n.a.	n.a.
DYF403S1b	7162	65	9.1	7.0–11.6	FM Y-STR	1931	22	11.4	4320	32	7.4	0.1381
DYS626	7910	68	8.6	6.7–10.9	FM Y-STR	2218	22	9.9	4320	35	8.1	0.4831
DYS458	11830	101	8.5	7.0–10.4	FM Y-STR	2555	22	8.6	7256	57	7.9	0.7005
DYS570	11717	97	8.3	6.7–10.1	FM Y-STR	2225	26	11.7	7764	50	6.4	0.0177
DYS385	11699	88	7.5	6.0–9.3	FM Y-STR	2561	15	5.9	7269	55	7.6	0.4153
DYS442	2025	15	7.4	4.2–12.2	FM Y-STR	2025	15	7.4	n.a.	n.a.	n.a.	n.a.
DYF393S1	2242	16	7.1	4.1–11.6	FM Y-STR	2242	16	7.1	n.a.	n.a.	n.a.	n.a.
DYS389II	11685	64	5.5	4.2–7.0	FM Y-STR	2542	8	3.1	7274	43	5.9	0.1091
DYS439	11687	56	4.8	3.6–6.2	MM Y-STR	2535	11	4.3	7282	35	4.8	0.8669
DYS481	8257	39	4.7	3.4–6.5	MM Y-STR	2543	12	4.7	5358	22	4.1	0.7145
DYS456	11488	50	4.4	3.2–5.7	MM Y-STR	2556	14	5.5	7274	27	3.7	0.2830
DYS460	8400	36	4.3	3.0–5.9	MM Y-STR	2887	15	5.2	5363	21	3.9	0.3879
DYS635	11472	44	3.8	2.8–5.1	MM Y-STR	2531	7	2.8	7283	32	4.4	0.3584
DYS533	8250	29	3.5	2.4–5.0	MM Y-STR	2529	14	5.5	5365	15	2.8	0.0723
DYS390	11070	30	2.7	1.8–3.9	MM Y-STR	2557	2	0.8	6644	19	2.9	0.0846
DYS391	11711	29	2.5	1.7–3.6	MM Y-STR	2558	9	3.5	7284	15	2.1	0.2416
DYS389I	11702	28	2.4	1.6–3.5	MM Y-STR	2550	10	3.9	7283	14	1.9	0.0999
DYS19	11707	23	2.0	1.2–2.9	MM Y-STR	2555	10	3.9	7283	11	1.5	**0.0417**
YGATAH4	11493	22	1.9	1.2–2.9	MM Y-STR	2554	8	3.1	7281	13	1.8	0.2154
DYS393	11702	20	1.7	1.0–2.6	MM Y-STR	2549	6	2.4	7284	10	1.4	0.3895
DYS437	11707	14	1.2	0.7–2	MM Y-STR	2559	4	1.6	7279	6	0.8	0.2972
DYS448	10735	9	0.8	0.4–1.6	SM Y-STR	2546	1	0.4	6531	4	0.6	1.0000
DYS392	11677	9	0.8	0.4–1.5	SM Y-STR	2527	1	0.4	7281	7	1.0	0.6888
DYS438	11702	3	0.3	0.1–0.7	SM Y-STR	2550	1	0.4	7283	2	0.3	1.0000

Data from 32 previous studies [2–4,15,16,19–43] were combined with current data, Table S2 shows the data per study.

be appropriate to use population-specific mutation rate estimates in future applications.

3.3. Differentiating related males

Next, we used the data to derive empirical father-son differentiation rates, defined as percentage of father-son pairs out of all pairs analyzed that differ by at least one Y-STR mutation. Overall, RMplex yielded a markedly higher father-son differentiation rate than Yfiler Plus did and considering all Y-STRs from both methods combined led to a further, albeit more modest, increase compared to RMplex.

With Yfiler Plus, 71 of 530 father-son pairs were differentiated, resulting in a father-son differentiation rate of 13.4% (Fig. 1). This differentiation rate is more than twice as high as previously obtained for the predecessor Yfiler which was 5.0% [2]. The increased differentiation rate of Yfiler Plus compared to Yfiler was expected because 10 more loci, including six RM Y-STRs, were included in Yfiler Plus, hence increased chance for mutations to be observed. However, the differentiation rate of Yfiler Plus was still lower than obtained with the 13 initially identified RM Y-STRs such as previously reported as 26.5% [2]. This is not unexpected because only six of the 13 RM Y-STRs are included in Yfiler Plus and because the effect of RM Y-STRs on father-son differentiation is stronger than that of Y-STRs with moderate mutation rates which Yfiler Plus consists of mostly.

Importantly, RMplex far outperformed Yfiler Plus with an estimated father-son differentiation rate of 41.9% (Fig. 1), with 209 out of 499 pairs being differentiated. The father-son differentiation rate obtained with RMplex was three times higher than that obtained with Yfiler Plus and almost 60% higher than with the initial set of 13 RM Y-STRs. Combining the data from RMplex and Yfiler Plus and considering the total of 49 Y-STRs, a further increase of the differentiation rate to 47.5% (237 of 499 pairs) was noted (Fig. 1). This increase of 5.6% points compared to RMplex alone is rather small when considering that a relatively large number of 19 Y-STRs in Yfiler Plus (not overlapping with

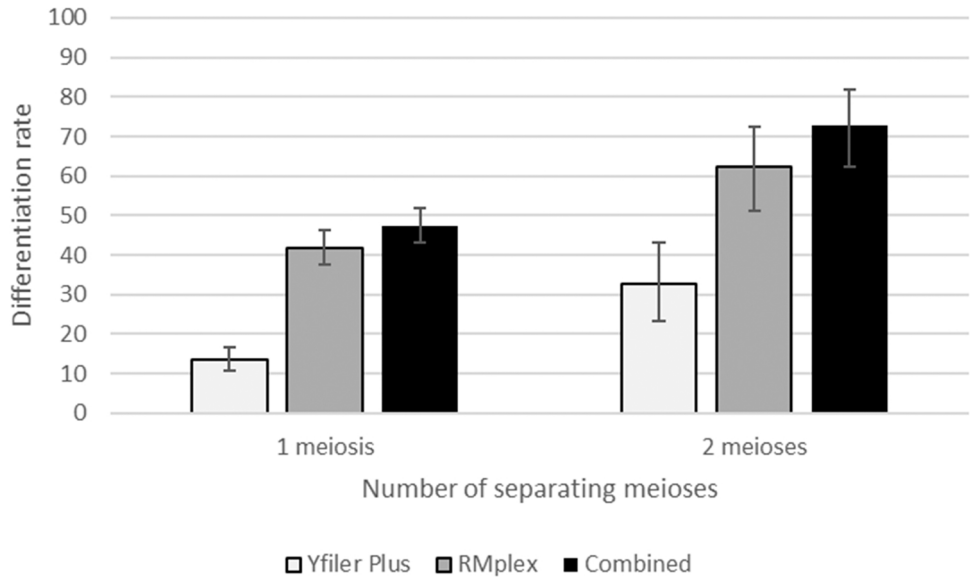

Fig. 1. : Male relative differentiation rates for father-son pairs (1 meiosis) and brother pairs (2 meioses) obtained with Yfiler Plus (25 Y-STRs), RMplex (30 Y-STRs), and both methods combined (49 Y-STRs). The error bars represent the exact binomial 95% confidence interval (Clopper-Pearson).

RMplex) was responsible for this. This reemphasizes once again that Y-STRs with moderate mutation rates can improve male relative differentiation, especially when being applied in larger numbers, but their effect is much smaller than that of Y-STRs with increased mutation rates, such as in RMplex.

Similarly, the differentiation rate for brother pairs were also improved strongly by both methods compared to those previously reported for sets with less Y-STRs. While Yfiler and the initial 13 RM Y-STRs previously differentiated 10.4% and 44.0% of brothers, respectively [2], based on the current data, Yfiler Plus and RMplex achieved a strong increase with differentiation rate estimates of 32.6% and 62.4%, respectively (Fig. 1). As expected, the superiority of RMplex over Yfiler Plus observed for father-son pairs is also evident for brother pairs. Combining both methods increased the brother differentiation rate further to 72.9%. This increase was higher than that seen for father-son pairs based on the combined marker set; however, in the current study, the sample size of brothers was with 92 pairs much smaller than that of father-son pairs. Therefore, the obtained brother differentiation rates are expected to be less reliable than those for father-son pairs, as illustrated by the larger error bars in Fig. 1. Therefore, until many more brother pairs are analyzed in the future, the brother differentiation rates reported here shall be treated with care.

Since given the definition used, the above-described male relative differentiation rates are based on one or more mutations, we also

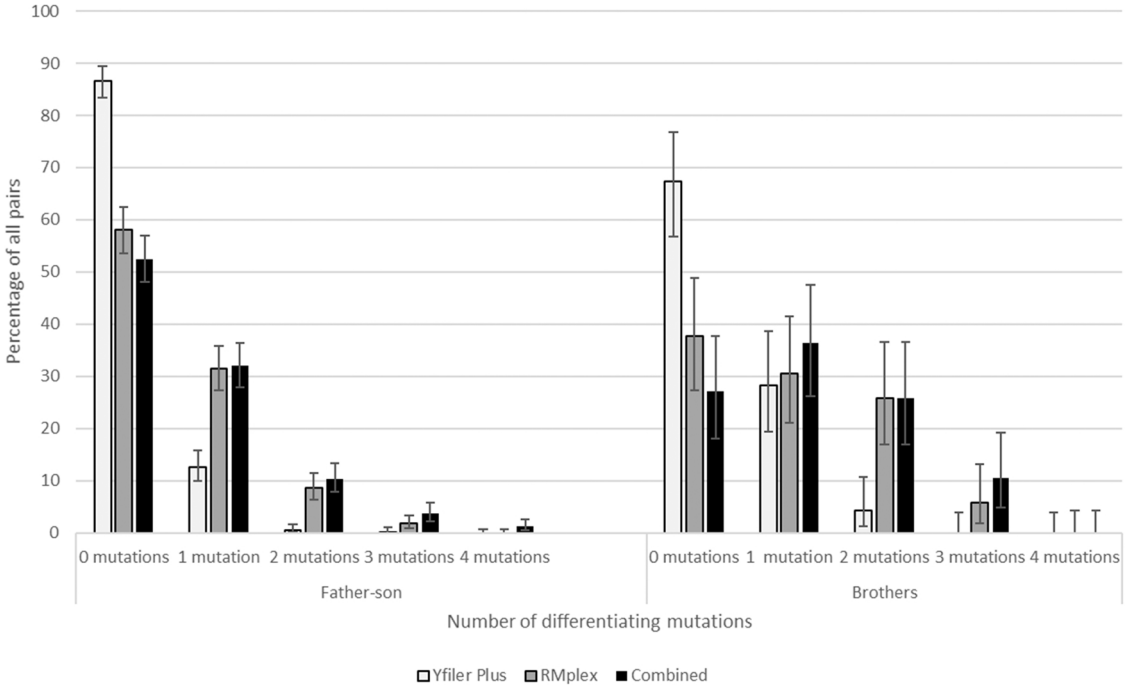

Fig. 2. Proportions of father-son pairs and brother pairs analyzed with Yfiler Plus (25 Y-STRs), RMplex (30 Y-STRs), and both methods combined (49 Y-STRs) with mutations at zero, one, two, three, and four Y-STR markers per pair. None of these pairs was differentiated by mutations at more than four markers. The error bars represent the exact binomial 95% confidence interval (Clopper-Pearson).

investigated the number of Y-STRs that showed a mutational difference between any given differentiated father-son or brother pair (Fig. 2, for illustrative reason we also included the pairs with zero mutations). Of all 209 father-son pairs differentiated with RMplex, 75.1% were separated by a single mutation at one Y-STR marker, 20.6% by mutations at two, and 4.3% at three markers. For the 71 father-son pairs differentiated with Yfiler Plus, the relative difference between the percentage of pairs differentiated by one mutation (94.4%), compared to those explained by mutations at two (4.2%) and three markers (1.4%), was larger than with RMplex, likely due to the lower number of Y-STRs with high mutation rates in Yfiler Plus. When considering all 49 Y-STRs targeted by both methods together, slightly more pairs were differentiated by more than a single mutation (Fig. 2). Notably, based on the combined analysis, six (2.5%) of the 237 differentiated father-son pairs showed mutations at four Y-STR markers, which was not seen with RMplex and Yfiler Plus alone. These data, demonstrating DNA-confirmed father-son pairs with mutations at 2–4 Y-STRs, provide relevant knowledge that shall be considered in interpreting results of Y-STR based paternity testing in deficiency cases where the mother of the disputed male child is unavailable for DNA testing and thus Y-STR testing is indicated.

Compared to father-son pairs, brother pairs separated by more than one mutation were seen more frequently for both methods separately and combined (Fig. 2), which may be explained by the double number of meiosis separating brothers relative to father-sons and thus double the opportunity for a mutation to arise at any given locus. In contrast to father-son pairs, we did not observe a brother pair with more than three mutations, which could be a result of the smaller sample size of brothers relative to father-son pairs. Observing more than a single mutation in brothers was a lot more common with RMplex compared to Yfiler Plus in both father-son pairs and brother pairs (Fig. 2).

3.4. Differentiating unrelated males

RM Y-STRs have previously been shown to not only increase the differentiation of related males, but also that of unrelated ones, e.g., when comparing results from the set of 13 initial RM Y-STRs with those from Yfiler targeting 16 Y-STRs in the same set of samples [4]. Since in the current study, markers were added to both sets, we additionally investigated the differentiation of the unrelated individuals. For RMplex, we obtained 469 unique haplotypes among 470 unrelated men of which 468 were found in a single man and one was shared between two men, resulting in a haplotype discrimination capacity of 99.8%. For Yfiler Plus, we observed 481 unique haplotypes among 482 unrelated men of which 480 were found in a single man and one was shared between two men, which also results in a haplotype discrimination capacity of 99.8%. The estimated haplotype diversity for both marker sets was also the same with 0.999991. Notably, the two men that shared the same 30-marker RMplex haplotype also shared the same 25-marker YFiler Plus haplotype. Sharing the same allele at 49 Y-STRs, of which the majority has high mutation rates, is unlikely to be found in two unrelated men and likely indicates a hidden relationship. As a result of the complete sample anonymization prior to this study, it could not be investigated to what degree these two men are paternally related, but our Y-STR results strongly suggest that they are paternally related and rather closely than distantly related.

A previous study based on the 13 initial RM Y-STRs and the 16 Yfiler Y-STRs [4] reported an increase in haplotype diversity with RM Y-STRs relative to Yfiler for three population samples from Austria i.e., Tyrol, Upper Australia, and Salzburg (13 RM Y-STRs: 0.99988, 0.99996, and 0.99995, respectively; Yfiler: 0.9996, 0.9998, and 0.9998 respectively [4]). Also, in these previously analyzed Austrian population samples, the haplotype discrimination capacities were increased considerably with RM Y-STRs relative to Yfiler (13 RM Y-STRs: 99.2%, 99.6%, and 99.5%, Yfiler: 97.7%, 97.3% and 98.1%). The increased haplotype diversity and discrimination capacity the current (an albeit different) Austrian population sample from Salzburg reveals for both Y-STR sets is expected given that both sets contain more Y-STRs relative to their counterparts used in the previous study.

What was not expected, however, is that the present study did not reveal any differences in discrimination capacity and haplotype diversity between RMplex and Yfiler Plus. It may be that this equal finding is influenced by sample size effects, as the more Y-STRs are analyzed, and especially the more markers with high mutation rates, the larger the population sample size needs to be to obtain reliable diversity estimates. Until data from more diverse populations and with larger sample size become available based on both methods, the present result of achieving the same differentiation of unrelated males with RMplex and Yfiler Plus shall be treated with care. In the future, it would be interesting to increase the sample size to see if the equal diversity measures obtained here for RMplex and Yfiler Plus remain or not. Our finding may also be influenced by the European population background of the samples analyzed here and future studies should investigate non-European populations. To allow future comparisons of the allele frequencies of these Y-STRs in different populations, we present the observed allele frequencies obtained from all fathers in the present study in Supplementary Table S3.

4. Conclusions

We present here the first application of RMplex and Yfiler Plus on a relatively large set of DNA-confirmed father-son pairs for obtaining empirical estimates of mutation rates, male relative differentiation rates for father-sons and for brothers, as well as haplotype diversity based on the unrelated men. The mutation rates achieved here were not significantly different from those previously obtained for these markers and the established updated reference mutation rate estimates are made available for future use. Father-son and brother differentiation rates are reported here for the first time for both marker sets and methods. Also the first time, we empirically demonstrate the improved differentiation of close male relatives achieved with RMplex compared to the current state-of-the-art commercial Y-STR kit: Yfiler Plus. With the increased number of RM Y-STRs included in RMplex, our study reaffirms the high value of RM Y-STRs for differentiating paternally related males. Future work should perform RMplex analysis in more distantly related males and in male relatives with more diverse paternal biogeographical backgrounds. Motivated by our findings, we encourage the forensic Y-chromosome community to use RMplex in all forensic cases where a match with any previously or currently available commercial Y-STR kit was obtained between the male suspect and the evidence material, or to solely use RMplex in suitable cases such as sexual assault cases, aiming to find out if the male suspect left the evidence material at the crime scene, or any of his male paternal relatives did.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2022.102682.

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